Fennema's Food Chemistry

Fourth Edition

CRC Press
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edited by
Srinivasan Damodaran
Kirk L. Parkin
Owen R. Fennema

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FOOD SCIENCE AND TECHNOLOGY

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Preface

Another decade has past since the publication of the third edition of *Food Chemistry*, and given the rapid progress in biological research, an update is warranted. However, this fourth edition marks several transitions. Perhaps, most important is the recognition of Owen Fennema's contributions to this text and to the field of food chemistry in general. His timely introduction of the first edition of *Food Chemistry* over 30 years ago, in 1976, filled a long-standing void of a comprehensive text that could serve as both an instructional tool and a desk reference for professionals. To us, it seems only fitting to now recognize this text as *Fennema's Food Chemistry*, as a tribute to his long-lasting contributions to the field through the three pervious editions of this text.

Since professor Fennema's "retirement" in 1996, he has remained professionally active, while engaging in more earthly pursuits of global travel, craftsmanship with wood, and stained glass artisanship. While he has been active with the planning of this edition as a coeditor, he entrusted us to assume most of the day-to-day editorial responsibilities. We are humbled, and needless to say that given the high standards set by professor Fennema in the previous editions, we are cognizant of the lofty expectations that likely exist for the fourth edition. Professor Fennema is a hard act to follow, and we hope our effort will not disappoint.

This edition not only marks a transition in editorial responsibilities, but also in contributing authors, as several former authors have retired or are approaching retirement. New (co)contributors appear for chapters on "Water and Ice," "Carbohydrates," "Lipids," "Enzymes," and "Colorants." Some chapters have also evolved in terms of focus and include "Postmortem Physiology of Edible Muscle Tissues," "Postharvest Physiology of Edible Plant Tissues," "Bioactive Substances: Nutraceuticals and Toxicants" (formerly "Toxic Substances"), and "Physical and Chemical Interactions of Components in Food Systems" (formerly "Summary: Integrative Concepts"), all with new (co)contributors. An added chapter appears on "Impact of Biotechnology on Food Supply and Quality."

We are indebted to the contributing authors of this volume for their patience and professionalism in dealing with new editors and for paying serious attention to the needs for chapter updates. It is hoped that both new and faithful readers of this text will find it useful, and be constructive by directing any comments regarding the content of this book (as well as identifying inevitable printing errors) to our attention.

Srinivasan Damodaran and Kirk Parkin
Madison, Wisconsin, USA

Editors

Owen R. Fennema is a professor of food chemistry in the Department of Food Science at the University of Wisconsin-Madison. He is coauthor of the books *Low Temperature Foods and Living Matter* (with William D. Powrie and Elmer H. Marth) and *Principles of Food Science, Part II: Physical Principles of Food Preservation* (with Marcus Karel and Daryl B. Lund), both titles published by Marcel Dekker, Inc., and the author or coauthor of over 175 professional papers that reflect his research interests in food chemistry, low-temperature preservation of food and biological matter, the characteristics of water and ice, edible films and coatings, and lipid–fiber interactions. A consulting editor for the *Food Science and Technology* series (Marcel Dekker, Inc.), he is a fellow of the Institute of Food Technologists and of the Agriculture and Food Chemistry Division of the American Chemical Society, and a member of the American Institute of Nutrition, among other organizations. Dr. Fennema received the BS degree (1950) in agriculture from Kansas State University, Manhattan, the MS degree (1951) in dairy science, and PhD degree (1960) in food science and biochemistry from the University of Wisconsin-Madison.

Sinivasan Damodaran is a professor of food chemistry and chair of the Department of Food Science at the University of Wisconsin-Madison. He is editor of the book *Food Proteins and Lipids* (Plenum Press) and co-editor of the book *Food Proteins and Their Applications* (with Alain Paraf) (Marcel Dekker, Inc.) and the author/coauthor of 6 patents and over 125 professional papers in his research areas, which include protein chemistry, enzymology, surface and colloidal science, process technologies, and industrial biodegradable polymers. He is a fellow of the Agriculture and Food Chemistry Division of the American Chemical Society and a member of the Institute of Food Science and the American Oil Chemists Society. He is on the editorial board of *Food Biophysics* journal. Dr. Srinivasan Damodaran received his BSc degree (1971) in chemistry from University of Madras, Madras, India, the MSc degree (1975) in food technology from Mysore University, Mysore, India, and PhD degree (1981) from Cornell University, Ithaca, New York.

Kirk L. Parkin is currently professor in the Department of Food Science of the University of Wisconsin (Madison, Wisconsin, USA), where he has been on the faculty for over 21 years. He has been the College of Agricultural and Life Sciences Fritz Friday Chair of Vegetable Processing Research since 1998, and was elected Fellow of the Agricultural and Food Chemistry Division of the American Chemical Society in 2003. Dr. Parkin's research and teaching interests revolve around food chemistry and biochemistry, with about 80 refereed journal publications in the areas of marine food biochemistry, postharvest physiology and processing of fruit and vegetable products, fundamental and applied enzymology, and most recently in the area of characterizing health-promoting and bioactive phytochemicals from foods of botanical origin. At UW-Madison, Dr. Parkin has been an instructor for undergraduate courses in Food Chemistry, Discovery Food Chemistry Laboratory, as well as for graduate courses in Food Enzymes and Lipids. He has supervised the completion of 10 Ph.D and 17 M.S. graduate degree programs, and serves as associate editor for *Journal of Food Science*, and on the editorial board of *Food Research International*, *Food Biochemistry*, and the *Journal of Food Processing and Preservation*.

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1 Introduction to Food Chemistry

Owen R. Fennema, Srinivasan Damodaran, and Kirk L. Parkin

CONTENTS

1.1 WHAT IS FOOD CHEMISTRY?

Food science deals with the physical, chemical, and biological properties of foods as they relate to stability, cost, quality, processing, safety, nutritive value, wholesomeness, and convenience. Food science is a branch of biological science and an interdisciplinary subject involving primarily microbiology, chemistry, biology, and engineering. Food chemistry, a major aspect of food science, deals with the composition and properties of food and the chemical changes it undergoes during handling, processing, and storage. Food chemistry is intimately related to chemistry, biochemistry, physiological chemistry, botany, zoology, and molecular biology. The food chemist relies heavily on knowledge of the aforementioned sciences to effectively study and control biological substances as sources of human food. Knowledge of the innate properties of biological substances and mastery of the means of manipulating them are common interests of both food chemists and biological scientists. The primary interests of biological scientists include reproduction, growth, and changes that biological substances undergo under environmental conditions that are compatible or marginally compatible with life. To the contrary, food chemists are concerned primarily with biological substances that are dead or dying (postharvest physiology of plants and postmortem physiology of muscle) and changes they undergo when exposed to a wide range of environmental conditions. For example, conditions suitable for sustaining residual life processes are of concern to food chemists during the marketing of fresh fruits and vegetables, whereas conditions incompatible with life processes are of major interest when long-term preservation of food is attempted. In addition, food chemists are concerned with the chemical properties of disrupted food tissues (flour, fruit and vegetable juices, isolated and modified constituents, and manufactured foods), single-cell sources of food (eggs and microorganisms), and one major biological fluid, milk. In summary, food chemists have much in common with biological scientists, yet they also have interests that are distinctly different and are of the utmost importance to humankind.

1.2 HISTORY OF FOOD CHEMISTRY

The origins of food chemistry are obscure, and details of its history have not yet been rigorously studied and recorded. This is not surprising, since food chemistry did not acquire a clear identity until the twentieth century, and its history is deeply entangled with that of agricultural chemistry for which historical documentation is not considered exhaustive [1,2]. Thus, the following brief excursion into the history of food chemistry is incomplete and selective. Nonetheless, available information is sufficient to indicate when, where, and why certain key events in food chemistry occurred and to relate some of these events to major changes in the wholesomeness of the food supply since the early 1800s.

Although the origin of food chemistry, in a sense, extends to antiquity, the most significant discoveries, as we judge them today, began in the late 1700s. The best accounts of developments during this period are those of Filby [3] and Browne [1], and these sources have been relied upon for much of the information presented here.

During the period of 1780–1850 a number of famous chemists made important discoveries, many of which related directly or indirectly to food, and these works contain the origins of modern food chemistry. Carl Wilhelm Scheele (1742–1786), a Swedish pharmacist, was one of the greatest chemists of all time. In addition to his more famous discoveries of chlorine, glycerol, and oxygen (3 years before Priestly, but unpublished), he isolated and studied the properties of lactose (1780), prepared mucic acid by oxidation of lactic acid (1780), devised a means of preserving vinegar by the application of heat (1782, well in advance of Appert's "discovery"), isolated citric acid from lemon juice (1784) and gooseberries (1785), isolated malic acid from apples (1785), and tested 20 common fruits for the presence of citric, malic, and tartaric acids (1785). His isolation of various new chemical compounds from plant and animal substances is considered the beginning of accurate analytical research in agricultural and food chemistry.

The French chemist Antoine Laurent Lavoisier (1743–1794) was instrumental in the final rejection of the phlogiston theory and in formulating the principles of modern chemistry. With respect to food chemistry, he established the fundamental principles of combustion organic analysis, he was the first to show that the process of fermentation could be expressed as a balanced equation, he made the first attempt to determine the elemental composition of alcohol (1784) and he presented one of the first papers (1786) on organic acids of various fruits.

(Nicolas) Théodore de Saussure (1767–1845), a French chemist, did much to formalize and clarify the principles of agricultural and food chemistry provided by Lavoisier. He also studied CO_2 and O_2 changes during plant respiration (1804) and the mineral contents of plants by ashing, and made the first accurate elemental analysis of alcohol (1807).

Joseph Louis Gay-Lussac (1778–1850) and Louis-Jacques Thenard (1777–1857) devised in 1811 the first method to determine percentages of carbon, hydrogen, and nitrogen in dry vegetable substances.

The English chemist Sir Humphrey Davy (1778–1829) in the years 1807 and 1808 isolated the elements K, Na, Ba, Sr, Ca, and Mg. His contributions to agricultural and food chemistry came largely through his books on agricultural chemistry, of which the first (1813) was *Elements of Agriculture Chemistry, in a Course of Lectures for the Board of Agriculture* [4]. His books served to organize and clarify knowledge existing at that time. In the first edition he stated,

> All the different parts of plants are capable of being decomposed into a few elements. Their uses as food, or for the purpose of the arts, depend upon compound arrangements of these elements, which are capable of being produced either from their organized parts, or from the juices they contain; and the examination of the nature of these substances is an essential part of agricultural chemistry.

In the fifth edition he stated that plants are usually composed of only seven or eight elements, and that [5] "the most essential vegetable substances consist of hydrogen, carbon, and oxygen in different proportion, generally alone, but in some few cases combined with azote [nitrogen]" (p. 121).

The works of the Swedish chemist Jons Jacob Berzelius (1779–1848) and the Scottish chemist Thomas Thomson (1773–1852) resulted in the beginnings of organic formulas, "without which organic analysis would be a trackless desert and food analysis an endless task" [3]. Berzelius determined the elemental components of about 2000 compounds, thereby verifying the law of definite proportions. He also devised a means of accurately determining the water content of organic substances, a deficiency in the method of Gay-Lussac and Thenard. Moreover, Thomson showed that laws governing the composition of inorganic substances apply equally well to organic substances, a point of immense importance.

In a book entitled *Considérations générales sur l'analyse organique et sur ses applications* [6], Michel Eugene Chevreul (1786–1889), a French chemist, listed the elements known to exist at that time in organic substances (O, Cl, I, N, S, P, C, Si, H, Al, Mg, Ca, Na, K, Mn, and Fe) and cited the processes then available for organic analysis: (1) extraction with a neutral solvent, such as water, alcohol, or aqueous ether; (2) slow distillation or fractional distillation; (3) steam distillation; (4) passing the substance through a tube heated to incandescence; and (5) analysis with oxygen. Chevreul was a pioneer in the analysis of organic substances, and his classic research on the composition of animal fat led to the discovery and naming of stearic and oleic acids.

Dr. William Beaumont (1785–1853), an American Army surgeon stationed at Fort Mackinac, MI, performed classic experiments on gastric digestion that destroyed the concept existing from the time of Hippocrates that food contained a single nutritive component. His experiments were performed during the period 1825–1833 on a Canadian, Alexis St. Martin, whose musket wound afforded direct access to the stomach interior, thereby enabling food to be introduced and subsequently examined for digestive changes [7].

Among his many notable accomplishments, Justus von Liebig (1803–1873) showed in 1837 that acetaldehyde occurs as an intermediate between alcohol and acetic acid during fermentation of vinegar. In 1842, he classified foods as either nitrogenous (vegetable fibrin, albumin, casein, and animal flesh and blood) or nonnitrogenous (fats, carbohydrates, and alcoholic beverages). Although this classification is not correct in several respects, it served to distinguish important differences among various foods. He also perfected methods for the quantitative analysis of organic substances, especially by combustion, and he published in 1847 what is apparently the first book on food chemistry, *Researches on the Chemistry of Food* [8]. Included in this book are accounts of his research on the water-soluble constituents of muscle (creatine, creatinine, sarcosine, inosinic acid, lactic acid, etc.).

It is interesting that the developments just reviewed paralleled the beginning of serious and widespread adulteration of food, and it is no exaggeration to state that the need to detect impurities in food was a major stimulus for the development of analytical chemistry in general and analytical food chemistry in particular. Unfortunately, it is also true that advances in chemistry contributed somewhat to the adulteration of food, since unscrupulous purveyors of food were able to profit from the availability of chemical literature, including formulas for adulterated food, and could replace older, less-effective empirical approaches to food adulteration with more efficient approaches based on scientific principles. Thus, the history of food chemistry and food adulteration are closely interwoven by the threads of several causative relationships, and it is therefore appropriate to consider the matter of food adulteration from a historical perspective [3].

The history of food adulteration in the currently more developed countries of the world falls into three distinct phases. From ancient times to about 1820 food adulteration was not a serious problem and there was little need for methods of detection. The most obvious explanation for this situation was that food was procured from small businesses or individuals and transactions involved a large measure of interpersonal accountability. The second phase began in the early 1800s, when intentional food adulteration increased greatly in both frequency and seriousness. This development can be attributed primarily to increased centralization of food processing and distribution, with a corresponding decline in interpersonal accountability, and partly to the rise of modern chemistry, as already mentioned. Intentional adulteration of food remained a serious problem until

about 1920, which marks the end of phase two and the beginning of phase three. At this point, regulatory pressures and effective methods of detection reduced the frequency and seriousness of intentional food adulteration to acceptable levels, and the situation has gradually improved up to the present time.

Some would argue that a fourth phase of food adulteration began about 1950, when foods containing legal chemical additives became increasingly prevalent, when the use of highly processed foods increased to a point where they represented a major part of the diet of persons in most of the industrialized countries, and when contamination of some foods with undesirable by-products of industrialization, such as mercury, lead, and pesticides, became of public and regulatory concern. The validity of this contention is hotly debated and disagreement persists to this day. Nevertheless, the course of action in the next few years seems clear. Public concern over the safety and nutritional adequacy of the food supply continues to evoke changes, both voluntary and involuntary, in the manner in which foods are produced, handled, and processed, and more such actions are inevitable as we learn more about proper handling practices for food and as estimates of maximum tolerable intake of undesirable constituents become more accurate.

The early 1800s was a period of especially intense public concern over the quality and safety of the food supply. This concern, or more properly indignation, was aroused in England by Frederick Accum's publication *A Treatise on Adulterations of Food* [9] and by an anonymous publication entitled *Death in the Pot* [10]. Accum claimed that "Indeed, it would be difficult to mention a single article of food which is not to be met with in an adulterated state; and there are some substances which are scarcely ever to be procured genuine" (p. 14). He further remarked, "It is not less lamentable that the extensive application of chemistry to the useful purposes of life, should have been perverted into an auxiliary to this nefarious traffic [adulteration]" (p. 20).

Although Filby [3] asserted that Accum's accusations were somewhat overstated, it was true that the intentional adulteration of several foods and ingredients prevailed in the 1800s, as cited by Accum and Filby, including annatto, black pepper, cayenne pepper, essential oils, vinegar, lemon juice, coffee, tea, milk, beer, wine, sugar, butter, chocolate, bread, and confectionary products.

Once the seriousness of food adulteration in the early 1800s was made evident to the public, remedial forces gradually increased. These took the form of new legislation to make adulteration unlawful, and greatly expanded efforts by chemists to learn about the native properties of foods, the chemicals commonly used as adulterants, and the means of detecting them. Thus, during the period 1820–1850, chemistry and food chemistry began to assume importance in Europe. This was possible because of the work of the scientists already cited, and was stimulated largely by the establishment of chemical research laboratories for young students in various universities and by the founding of new journals for chemical research [1]. Since then, advances in food chemistry have continued at an accelerated pace, and some of these advances, along with causative factors, are mentioned below.

In 1860, the first publicly supported agriculture experiment station was established in Weede, Germany, and W. Hanneberg and F. Stohmann were appointed director and chemist, respectively. Based largely on the work of earlier chemists, they developed an important procedure for the routine determination of major constituents in food. By dividing a given sample into several portions they were able to determine moisture content, "crude fat," ash, and nitrogen. Then, by multiplying the nitrogen value by 6.25, they arrived at its protein content. Sequential digestion with dilute acid and dilute alkali yielded a residue termed "crude fiber." The portion remaining after removal of protein, fat, ash, and crude fiber was termed "nitrogen-free extract," and this was believed to represent utilizable carbohydrate. Unfortunately, for many years chemists and physiologists wrongfully assumed that like values obtained by this procedure represented like nutritive value, regardless of the kind of food [11].

In 1871, Jean Baptiste Duman (1800–1884) suggested that a diet consisting of only protein, carbohydrate, and fat was inadequate to support life.

In 1862, the Congress of the United States passed the Land-Grant College Act, authored by Justin Smith Morrill. This act helped establish colleges of agriculture in the United States and provided

considerable impetus for the training of agricultural and food chemists. Also in 1862, the U.S. Department of Agriculture was established and Isaac Newton was appointed the first commissioner.

In 1863, Harvey Washington Wiley became chief chemist of the U.S. Department of Agriculture, from which office he led the campaign against misbranded and adulterated food, culminating in passage of the first Pure Food and Drug Act in the United States (1906).

In 1887, agriculture experiment stations were established in the United States following enactment of the Hatch Act. Representative William H. Hatch of Missouri, Chairman of the House Committee on Agriculture, was author of the act. As a result, the world's largest national system of agriculture experiment stations came into existence and this had a great impact on food research in the United States.

During the first half of the twentieth century, most of the essential dietary substances were discovered and characterized, namely, vitamins, minerals, fatty acids, and some amino acids.

The development and extensive use of chemicals to aid in the growth, manufacture, and marketing of foods was an especially noteworthy and contentious event in the mid-1900s.

This historical review, although brief, makes the current food supply seem almost perfect in comparison to that which existed in the 1800s. However, at this writing, several current issues have replaced the historical ones in terms of what the food science community must address in further promoting the wholesomeness and nutritive value of foods, while mitigating the real or perceived threats to the safety of the food supply. These issues include the nature, efficacy, and impact of nonnutrient components in foods, dietary supplements, and botanicals that can promote human health beyond simple nutrition (Chapter 12); molecular engineering of crops (genetically modified organisms or GMOs) and the benefits juxtaposed against the perceived risks to safety and human health (Chapter 18); and the comparative nutritive value of crops raised by organic vs. conventional agricultural methods.

1.3 APPROACH TO THE STUDY OF FOOD CHEMISTRY

Food chemists are typically concerned with identifying the molecular determinants of material properties and chemical reactivity of food matrices, and how this understanding is effectively applied to improve formulation, processing, and storage stability of foods. An ultimate objective is to determine cause-and-effect and structure–function relationships among different classes of chemical components. The facts derived from the study of one food or model system can be applied to our understanding of other food products. An analytical approach to food chemistry includes four components, namely: (1) determining those properties that are important characteristics of safe, high-quality foods; (2) determining those chemical and biochemical reactions that have important influences on loss of quality and/or wholesomeness of foods; (3) integrating the first two points so that one understands how the key chemical and biochemical reactions influence quality and safety; and (4) applying this understanding to various situations encountered during formulation, processing, and storage of food.

Safety is the first requisite of any food. In a broad sense, this means a food must be free of any harmful chemical or microbial contaminant at the time of its consumption. For operational purposes this definition takes on a more applied form. In the canning industry, "commercial" sterility as applied to low-acid foods means the absence of viable spores of *Clostridium botulinum*. This in turn can be translated into a specific set of heating conditions for a specific product in a specific package. Given these heating requirements, one can then select specific time–temperature conditions that will optimize retention of quality attributes. Similarly, in a product such as peanut butter, operational safety can be regarded primarily as the absence of aflatoxins—carcinogenic substances produced by certain species of molds. Steps taken to prevent growth of the mold in question may or may not interfere with retention of some other quality attribute; nevertheless, conditions producing a safe product must be employed.

A list of quality attributes of food and some alterations they can undergo during processing and storage is given in Table 1.1. The changes that can occur, with the exception of those involving nutritive value and safety, are readily evident to the consumer.

Many chemical and biochemical reactions can alter food quality or safety. Some of the more important classes of these reactions are listed in Table 1.2. Each reaction class can involve different reactants or substrates depending on the specific food and the particular conditions for handling,

TABLE 1.1
Classification of Alterations That Can Occur During Handling, Processing, or Storage

Attribute	Alteration
Texture	Loss of solubility
	Loss of water-holding capacity
	Toughening
	Softening
Flavor	Development of
	rancidity (hydrolytic or oxidative)
	cooked or caramel flavors
	other off-flavors
	desirable flavors
Color	Darkening
	Bleaching
	Development of desirable colors (e.g., browning of baked goods)
Nutritive value	Loss, degradation, or altered bioavailability of proteins, lipids, vitamins, minerals, and other health-promoting components
Safety	Generation of toxic substances
	Development of substances that are protective to health
	Inactivation of toxic substances

TABLE 1.2
Some Chemical and Biochemical Reactions That Can Lead to Alteration of Food Quality or Safety

Types of Reaction	Examples
Nonenzymic browning	Baked goods, dry, and intermediate moisture foods
Enzymic browning	Cut fruits and some vegetables
Oxidation	Lipids (off-flavors), vitamin degradation, pigment decoloration, proteins (loss of nutritive value)
Hydrolysis	Lipids, proteins, vitamins, carbohydrates, pigments
Metal interactions	Complexation (anthocyanins), loss of Mg from chlorophyll, catalysis of oxidation
Lipid isomerization	*cis* → *trans* isomerization, nonconjugated → conjugated
Lipid cyclization	Monocyclic fatty acids
Lipid oxidation–polymerization	Foaming during deep-fat frying
Protein denaturation	Egg white coagulation, enzyme inactivation
Protein crosslinking	Loss of nutritive value during alkali processing
Polysaccharide synthesis and degradation	In plants postharvest
Glycolytic changes	Animal postmortem, plant tissue postharvest

TABLE 1.3
Examples of Cause-and-Effects Relationships Pertaining to Food Alteration During Handling, Storage, and Processing

Primary Causative Event	Secondary Event	Attribute Influenced (see Table 1.1)
Hydrolysis of lipids	Free fatty acids react with protein	Texture, flavor, nutritive value
Hydrolysis of polysaccharides	Sugars react with protein	Texture, flavor, color, nutritive value
Oxidation of lipids	Oxidation products react with many other constituents	Texture, flavor, color, nutritive value; toxic substances can be generated
Bruising of fruit	Cells break, enzymes are released, oxygen accessible	Texture, flavor, color, nutritive value
Heating of horticultural products	Cell walls and membranes lose integrity, acids are released, enzymes become inactive	Texture, flavor, color, nutritive value
Heating of muscle tissue	Proteins denature and aggregate, enzyme become inactive	Texture, flavor, color, nutritive value
cis → *trans* conversion in lipids	Enhanced rate of polymerization during deep-fat frying	Excessive foaming during deep-fat frying, diminished nutritive value and bioavailability of lipids, solidification of frying oil

processing, or storage. They are treated as reaction classes because the general nature of the substrates or reactants is similar for all foods. Thus, nonenzymic browning involves reaction of carbonyl compounds, which can arise from existing reducing sugars or from diverse reactions, such as oxidation of ascorbic acid, hydrolysis of starch, or oxidation of lipids. Oxidation may involve lipids, proteins, vitamins, or pigments, and more specifically, oxidation of lipids may involve triacylglycerols in one food or phospholipids in another. Discussion of these reactions in detail will occur in subsequent chapters of this book.

The reactions listed in Table 1.3 cause the alterations listed in Table 1.1. Integration of the information contained in both tables can lead to an understanding of the causes of food deterioration. Deterioration of food usually consists of a series of primary events followed by secondary events, which, in turn, become evident as altered quality attributes (Table 1.1). Examples of sequences of this type are shown in Table 1.3. Note particularly that a given quality attribute can be altered as a result of several different primary events.

The sequences in Table 1.3 can be applied in two directions. Operating from left to right, one can consider a particular primary event, the associated secondary events, and the effect on a quality attribute. Alternatively, one can determine the probable cause(s) of an observed quality change (column 3, Table 1.3) by considering all primary events that could be involved and then isolating, by appropriate chemical tests, the key primary event. The utility of constructing such sequences is that they encourage one to approach problems of food alteration in an analytical manner.

Figure 1.1 is a simplistic summary of reactions and interactions of the major constituents of food. The major cellular pools of carbohydrates, lipids, proteins, and their intermediary metabolites are shown on the left-hand side of the diagram. The exact nature of these pools is dependent on the physiological state of the tissue at the time of processing or storage, and the constituents present in or added to nontissue foods. Each class of compound can undergo its own characteristic type of deterioration. Noteworthy is the role that carbonyl compounds play in many deterioration processes. They arise mainly from lipid oxidation and carbohydrate degradation and can lead to the destruction of nutritional value, to off-colors, and to off-flavors. Of course, these same reactions lead to desirable flavors and colors during the cooking of many foods.

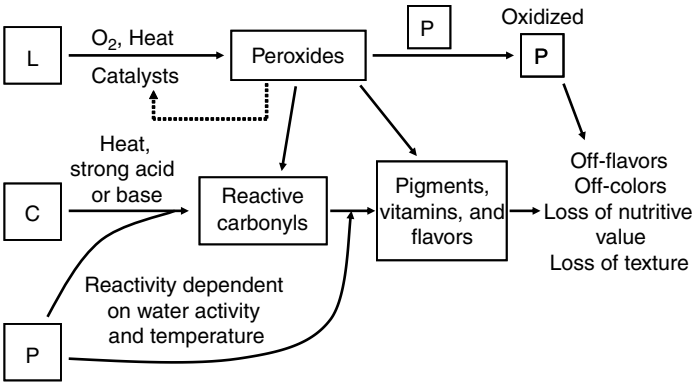

FIGURE 1.1 Summary of chemical interactions among major food constituents: L, lipid pool (triacylglycerols, fatty acids, and phospholipids); C, carbohydrate pool (polysaccharides, sugars, organic acids, etc.); P, protein pool (proteins, peptides, amino acids, and other N-containing substances).

TABLE 1.4
Important Factors Governing the Stability of Foods During Handling, Processing, and Storage

Product Factors	Environmental Factors
Chemical properties of individual constituents (including catalysts), oxygen content, pH water activity, T_g, and W_g	Temperature (T); time (t); composition of the atmosphere; chemical, physical, or biological treatments imposed; exposure to light; contamination; physical abuse

Note: Water activity $= p/p_o$, where p is the partial pressure of water vapor above the food and p_o is the vapor pressure of pure water; T_g is the glass transition temperature; W_g is the product water content at T_g.

1.3.1 Analysis of Situations Encountered During the Storage and Processing of Food

Having before us a description of the attributes of high-quality, safe foods, the significant chemical reactions involved in the deterioration of food, and the relationship between the two, we can now begin to consider how to apply this information to situations encountered during the storage and processing of food.

The variables that are important during the storage and processing of food are listed in Table 1.4. Temperature is perhaps the most important of these variables because of its broad influence on all types of chemical reactions. The effect of temperature on an individual reaction can be estimated from the Arrhenius equation, $k = Ae^{-\Delta E/RT}$. Data conforming to the Arrhenius equation yield a straight line when $\log k$ is plotted vs. $1/T$. The parameter ΔE is the activation energy that represents the free energy change required to elevate a chemical entity from a ground state to transition state, whereupon reaction can occur. Arrhenius plots in Figure 1.2 represent reactions important in food deterioration. It is evident that food reactions generally conform to the Arrhenius relationship over a limited intermediate temperature range but that deviations from this relationship can occur at high or low temperatures [12]. Thus, it is important to remember that the Arrhenius relationship for food systems is valid only over a range of temperature that has been experimentally verified. Deviations from the Arrhenius relationship can occur because of the following events, most of which are induced

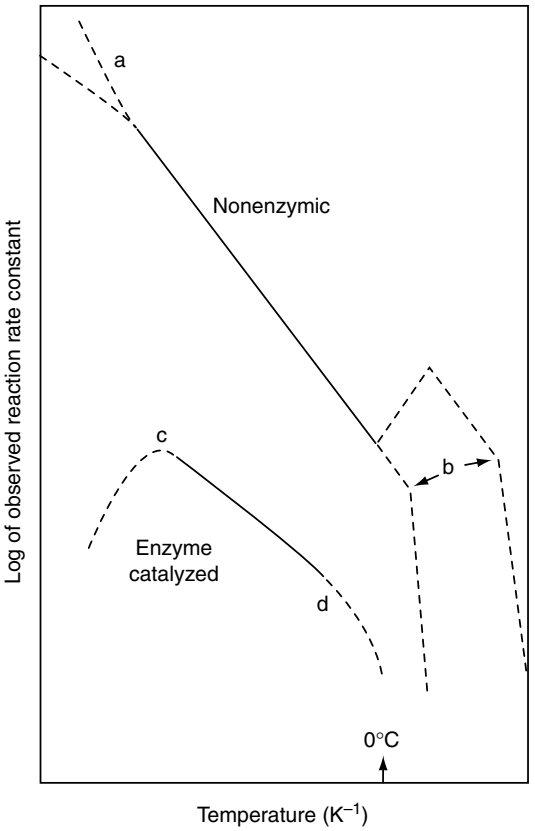

FIGURE 1.2 Conformity of important deteriorative reactions in food to the Arrhenius relationship. (a) Above a certain value of T there may be deviations from linearity due to a change in the path of the reaction. (b) As the temperature is lowered below the freezing point of the system, the ice phase (essentially pure) enlarges and the fluid phase, which contains all the solutes, diminishes. This concentration of solutes in the unfrozen phase can decrease reaction rates (supplement the effect of decreasing temperature) or increase reaction rates (oppose the effect of declining temperature), depending on the nature of the system (see Chapter 2). (c) For an enzymic reaction there is a temperature in the vicinity of the freezing point of water where subtle changes, such as the dissociation of an enzyme complex, can lead to a sharp decline in reaction rate.

by either high or low temperatures: (1) enzyme activity may be lost, (2) the reaction pathway or rate-limiting step may change or may be influenced by a competing reaction(s), (3) the physical state of the system may change (e.g., by freezing), or (4) one or more of the reactants may become depleted.

Another important factor in Table 1.4 is time. During storage of a food product, one frequently wants to know how long the food can be expected to retain a specified level of quality. Therefore, one is interested in time with respect to the integral of chemical and/or microbiological changes that occur during a specified storage period, and in the way these changes combine to determine a specified storage life for the product. During processing, one is often interested in the time it takes to inactivate a particular population of microorganisms or in how long it takes for a reaction to proceed to a specified extent. For example, it may be of interest to know how long it takes to produce a desired brown color in potato chips during frying. To accomplish this, attention must be given to temperature change with time, that is, dT/dt. This relationship is important because it allows the determination of the extent to which the reaction rate changes as temperature of the food matrix changes during the course of processing. If ΔE of the reaction and temperature profile of the food

are known, an integrative analysis affords a prediction of the net accumulation of reaction product. This is also of interest in foods that deteriorate by more than one means, such as lipid oxidation and nonenzymic browning. If the products of the browning reaction are antioxidants, it is important to know whether the relative rates of these reactions are such that a significant interaction will occur between them.

Another variable, pH, influences the rates of many chemical and enzymic reactions. Extreme pH values are usually required for severe inhibition of microbial growth or enzymic processes and these conditions can result in acceleration of acid- or base-catalyzed reactions. In contrast, even a relatively small pH change can cause profound changes in the quality of some foods, for example, muscle.

The composition of the product is important since this determines the reactants available for chemical transformation. Also important is how cellular vs. noncellular and homogenous vs. heterogenous food systems influence the disposition and reactivity of reactants. Particularly important from a quality standpoint is the relationship that exists between composition of the raw material and composition of the finished product. For example, (1) the manner in which fruits and vegetables are handled postharvest can influence sugar content, and this, in turn, influences the degree of browning obtained during dehydration or deep-fat frying; (2) the manner in which animal tissues are handled postmortem influences the extents and rates of glycolysis and ATP degradation, and these in turn can influence storage life, water-holding capacity, toughness, flavor, and color; and (3) the blending of raw materials may cause unexpected interactions for example, the rate of oxidation can be accelerated or inhibited depending on the amount of salt present.

Another important compositional determinant of reaction rates in foods is water activity (a_w). Numerous investigators have shown a_w to strongly influence the rate of enzyme-catalyzed reactions [13], lipid oxidation [14,15], nonenzymic browning [16,14], sucrose hydrolysis [17], chlorophyll degradation [18], anthocyanin degradation [19], and others. As is discussed in Chapter 2, most reactions tend to decrease in rate below an a_w corresponding to the range of intermediate moisture foods (0.75–0.85). Oxidation of lipids and associated secondary effects, such as carotenoid decoloration, are exceptions to this rule; that is, these reactions accelerate at the lower end of the a_w scale.

More recently, it has become apparent that the glass transition temperature (T_g) of food and the corresponding water content (W_g) at T_g are causatively related to rates of diffusion-limited events in the food. Thus, T_g and W_g have relevance to the physical properties of frozen and dried foods, to conditions appropriate for freeze drying, to physical changes involving crystallization, recrystallization, gelatinization, and starch retrogradation, and to those chemical reactions that are diffusion-limited (see Chapter 2).

In fabricated foods, the composition can be controlled by adding approved chemicals, such as acidulants, chelating agents, flavors, or antioxidants, or by removing undesirable reactants, for example, removing glucose from dehydrated egg albumen.

Composition of the atmosphere is important mainly with respect to relative humidity and oxygen content, although ethylene and CO_2 are also important during storage of living plant foods. Unfortunately, in situations where exclusion of oxygen is desirable, this is almost impossible to achieve completely. The detrimental consequences of a small amount of residual oxygen sometimes become apparent during product storage. For example, early formation of a small amount of dehydroascorbic acid (from oxidation of ascorbic acid) can lead to Maillard browning during storage.

For some products, exposure to light can be detrimental and it is then appropriate to package the products in light-impervious material or to control the intensity and wavelengths of light, if possible.

Food chemists must be able to integrate information about quality attributes of foods, deteriorative reactions to which foods are susceptible, and the factors governing kinds and rates of these deteriorative reactions, in order to solve problems related to food formulation, processing, and storage stability.

1.4 SOCIETAL ROLE OF FOOD CHEMISTS

1.4.1 Why Should Food Chemists Become Involved in Societal Issues?

Food chemists, for the following reasons, should feel obligated to become involved in societal issues that encompass pertinent technological aspects (technosocietal issues):

- Food chemists have had the privilege of receiving a high level of education and of acquiring special scientific skills, and these privileges and skills carry with them a corresponding high level of responsibility.
- Activities of food chemists influence adequacy of the food supply, healthfulness of the population, cost of foods, waste creation and disposal, water and energy use, and the nature of food regulations. Because these matters impinge on the general welfare of the public, it is reasonable that food chemists should feel a responsibility to have their activities directed to the benefit of society.
- If food chemists do not become involved in technosocietal issues, the opinions of others—scientists from other professions, professional lobbyists, persons in the news media, consumer activists, charlatans, antitechnology zealots—will prevail. Many of these individuals are less qualified than food chemists to speak on food-related issues and some are obviously unqualified.
- Food chemists have a role and opportunity to help resolve controversies that impact, or are perceived to impact, on public health and how the public views developments in science and technology. Examples of some current controversies include safety of cloned and GMOs, the use of animal growth hormones in agricultural production, and the relative nutritive value of crops produced through organic and conventional agricultural methods.

1.4.2 Types of Involvement

The societal obligations of food chemists include good job performance, good citizenship, and guarding the ethics of the scientific community, but fulfillment of these very necessary roles is not enough. An additional role of great importance, and one that often goes unfulfilled by food chemists, is that of helping determine how scientific knowledge is interpreted and used by society. Although food chemists and other food scientists should not have the only input to these decisions, they must, in the interest of wise decision making, have their views heard and considered. Acceptance of this position, which is surely indisputable, leads to the obvious question, "What exactly should food chemists do to properly discharge their responsibilities in this regard?" Several activities are appropriate:

- Participate in pertinent professional societies
- Serve on governmental advisory committees, when invited
- Undertake personal initiatives of a public service nature

The third point can involve letters to newspapers, journals, legislators, government regulators, company executives, university administrators, and others, and speeches dialog with civic groups, including sessions with K-12 students and all other stakeholders.

The major objectives of these efforts are to educate and enlighten the public with respect to food and dietary practices. This involves improving the public's ability to intelligently evaluate information on these topics. Accomplishing this will not be easy because a significant portion of the populace has ingrained false notions about food and proper dietary practices, and because food has, for many individuals, connotations that extend far beyond the chemist's narrow view. For these individuals, food may be an integral part of religious practice, cultural heritage, ritual,

social symbolism, or a route to physiological well-being—attitudes that are, for the most part, not conducive to acquiring an ability to appraise foods and dietary practices in a sound, scientific manner.

One of the most contentious food issues and one that has eluded appraisal by the public in a sound, scientific manner, is the use of chemicals to modify foods. "Chemophobia," the fear of chemicals, has afflicted a significant portion of the populace, causing food additives, in the minds of many, to represent hazards inconsistent with fact. One can find, with disturbing ease, articles in the popular literature whose authors claim the American food supply is sufficiently laden with poisons to render it unwholesome at best, and life-threatening at worst. Truly shocking, they say, is the manner in which greedy industrialists poison our foods for profit while an ineffectual Food and Drug Administration watches with placid unconcern. Should authors holding this viewpoint be believed? The answer to this question resides largely with how credible and authoritative the author is regarding the scientific issue at the center of debate. Credibility is founded on formal education, training, and practical experience, and scholarly contributions to the body of knowledge to which a particular dispute is linked. Scholarly activity can take the form of research, discovery of new knowledge, and the review and/or interpretation of a body of knowledge. Credibility is also founded on the author making all attempts to be objective, which requires consideration of alternative points of view and as much as the existing knowledge on the subject as feasible, instead of only pointing out facts and interpretations that are supportive of a preferred viewpoint. Knowledge accumulates through the publication of results of studies in the scientific literature, which is subject to peer-review and is held to specific professional standards of protocol, documentation, and ethics, thereby making them more authoritative than publications in the popular press.

Closer to the daily realm of the student or developing food science professional, a contemporary issue regarding the credibility of information deals with the expanse of information (including that of scientific nature) that is readily and easily accessible through the World Wide Web. Some such information is rarely attributed to any author, and the website may be void of obvious credentials to be regarded as a credible, authoritative source. Some information may be posted to advance a preferred point of view or cause, or be part of a marketing campaign to influence the viewer's thinking or purchasing habits. While some information on the web is as authoritative as media disseminated by trained scientists and scientific publishers, the student is encouraged to carefully consider the source of information obtained from the World Wide Web and not simply defer to the expedience in accessing it.

Despite the current and growing expanse of knowledge in food science, disagreement about the safety of foods and other food science issues still occurs. The great majority of knowledgeable individuals support the view that our food supply is acceptably safe and nutritious and that legally sanctioned food additives pose no unwarranted risks [20–30], although continued vigilance for adverse effects is warranted. However, a relatively small group of knowledgeable individuals believe that our food supply is unnecessarily hazardous, particularly with regard to some of the legally sanctioned food additives.

Scientific debate in public forums has more recently expanded to include the public and environmental safety of GMOs, the relative nutritive value of organic and conventionally grown crops, and the appropriateness of marketing-driven statements that the public may construe as health claims accompanying dietary supplements, among others. Scientific knowledge develops incrementally and at a slower rate than can fully prepare us for the next debate. It is the scientists' role to be involved in the process and encourage the various parties to focus objectively on the science and knowledge, enabling fully informed policy makers to reach an appropriate conclusion.

In summary, scientists have greater obligations to society than do individuals without formal scientific education. Scientists are expected to generate knowledge in a productive and ethical manner, but this is not enough. They should also accept the responsibility of ensuring that scientific knowledge is used in a manner that will yield the greatest benefit to society. Fulfillment of this obligation requires that scientists not only strive for excellence and conformance to high ethical standards in

their day-to-day professional activities, but that they also develop a deep-seated concern for the well-being and scientific enlightenment of the public.

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Part I

Major Food Components

2 Water and Ice

David S. Reid and Owen R. Fennema

CONTENTS

2.1 INTRODUCTION

When we examine the composition of most foods, water is found to be a substantial component. Also, when we consider our own metabolic processes, water is the primary solvent in which these life processes occur. It is therefore appropriate to delve into the nature and properties of water and aqueous solutions, and to consider the many roles played by water in food systems in order to understand the central role of water in food chemistry.

2.2 THE PHYSICAL PROPERTIES OF WATER AND ICE

As a first step in becoming familiar with water, it is appropriate to consider its physical properties, as shown in Table 2.1. By comparing water's properties with those of molecules of similar molecular weight and atomic composition (Table 2.2), it is possible to determine whether water behaves in a normal fashion or whether its behavior is unusual. On the basis of these comparisons [1], water is seen to have unusually high melting and boiling point temperatures, to exhibit unusually large values for surface energy, permittivity, heat capacity, and heats of phase transformation (fusion, vaporization, and sublimation), to have a somewhat lower than expected density, to exhibit the unusual property of expansion upon solidification, and yet, despite these unusual properties, to have a viscosity that is quite normal. This apparent normality for a clearly anomalous liquid will be explained later.

Other properties of water are also remarkable. The thermal conductivity of water is large as compared with most other liquids, and the thermal conductivity of ice is larger than might be expected for a nonmetallic solid. It is noteworthy that the thermal conductivity of ice at 0°C is approximately quadruple that of liquid water at the same temperature, indicating that ice will conduct thermal energy at a much greater rate than will immobilized (e.g., tissue) water. Since the heat capacity of water is approximately twice that of ice, the thermal diffusivities of water and ice differ by about a factor of 9 [2]. Since thermal diffusivity is indicative of the rate at which a material will undergo a change in temperature, we would expect that ice, in a given thermal environment, will undergo temperature change at a rate 9 times greater than that for liquid water. These differences in thermal conductivity and diffusivity values for water and ice provide a good basis for understanding why tissues freeze more rapidly than they thaw under symmetrically applied temperature differentials [2].

2.3 THE WATER MOLECULE

The unusual properties of water suggest that strong attractive forces exist among water molecules and also suggest that the structures of water and ice might be unusual. To explain the features and

TABLE 2.1
Physical Properties of Water and Ice

Property	Value
Molecular weight	18.0153
Melting point (at 101.3 kPa)	0.00°C
Boiling point (at 101.3 kPa)	100.00°C
Critical temperature	373.99°C
Critical pressure	22.064 Mpa
Triple point temperature	0.01°C
Triple point pressure	611.73 Pa
ΔH_{vap} at 100°C	40.647 kJ/mol
ΔH_{sub} at 0°C	50.91 kJ/mol
ΔH_{fus} at 0°C	6.002 kJ/mol

	Temperature (°C)			
	Ice		Water	
Other Temperature-Dependent Properties	**−20**	**0**	**0**	**+20**
Density (g/cm^3)	0.9193	0.9168	0.99984	0.99821
Vapor pressure (kPa)	0.103	0.6113	0.6113	2.3388
Heat capacity (J/g/K)	1.9544	2.1009	4.2176	4.1818
Thermal conductivity (W/m/K)	2.433	2.240	0.561	0.5984
Thermal diffusivity (m^2/s)	11.8×10^{-7}	11.7×10^{-7}	1.3×10^{-7}	1.4×10^{-7}
Compressibility (Pa^{-1})		2	4.9	
Permittivity	98	90	87.9	80.2

Source: Lide, D.R. (Ed.) (1993/1994) *Handbook of Chemistry and Physics*, 74 edn. CRC Press: Boca Raton, FL.

TABLE 2.2
Properties of Related Small Molecules

	CH_4	NH_3	H_2O	H_2S	H_2Se	HF
MW	16.04	17.0	18.01	34.08	80.9	20.01
mp (°C)	−182.6	−77.7	0	−86	−60	−83.1
bp (°C)	−161.4	−33.3	100	−61	−41	19.5
ΔH_v (kJ/mol)	8.16	23.26	40.71	18.66		

Source: Lide, D.R. (Ed.) (1993/1994) *Handbook of Chemistry and Physics*, 74 edn. CRC Press: Boca Raton, FL.

unusual behavior of water and ice, it is best first to consider the nature of a single water molecule, and then to consider the characteristics of clusters of water molecules of increasing size, before finally considering the nature of the bulk system. The water molecule is often described as comprised of two hydrogen atoms interacting with the two sp^3 bonding orbitals of oxygen, forming two covalent sigma (σ) bonds of 40% ionic character, each of which has a dissociation energy of 4.6×10^2 kJ/mol. The localized molecular orbitals are assumed to remain symmetrically oriented about the original orbital axes, hence retaining an approximate tetrahedral structure. A schematic model is shown in

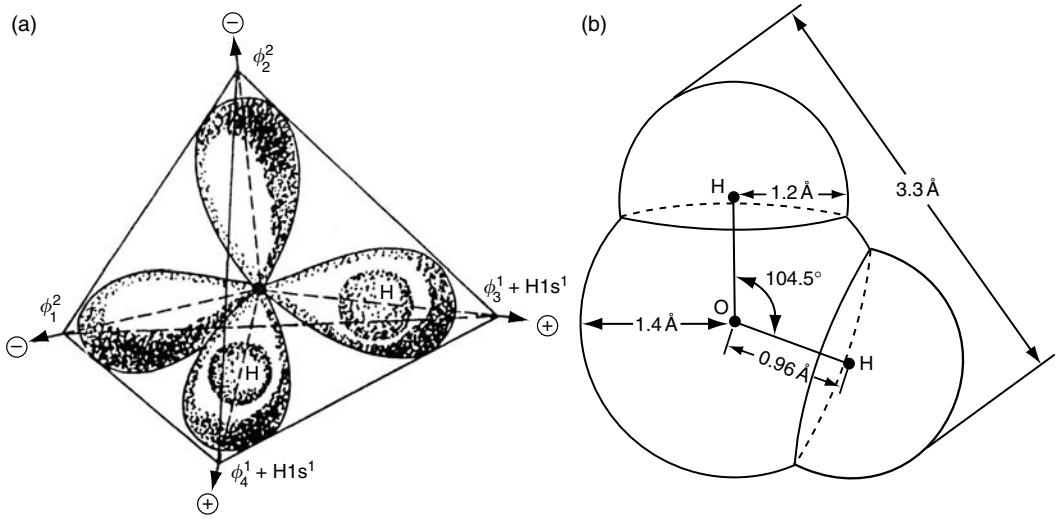

FIGURE 2.1 Schematic model of a single HOH molecule. (a) Possible sp^3 configuration and (b) van der Waals radii for a HOH molecule in vapor state.

Figure 2.1a and the van der Waals radii in Figure 2.1b. While the geometric behavior of water molecules associated through hydrogen bonding is consistent with this model, the assumption of extensive sp^3 hybridization of the lone pairs has been increasingly challenged [3].

In the vapor state, the bond angle of an isolated water molecule is 104.5° close to the perfect tetrahedral angle of 109.5° and the van der Waals radii for oxygen and hydrogen are, respectively, 1.40 and 1.2 Å [4].

At this point, it is important to note that the picture presented so far, describing only the HOH molecule, is oversimplified. The material we know as pure water is a mixture of HOH molecules and many other related constituents. In addition to the common isotopes of oxygen and hydrogen, ^{16}O and ^{1}H, also present are ^{17}O, ^{18}O, ^{2}H (D), and ^{3}H (T) with a resultant 18 isotopic variants of molecular HOH. Additionally, water contains ionic species such as hydrogen ions (existing in forms such as H_3O^+, $H_9O_4^+$) and hydroxyl ions, also with their isotopic variants. "Pure" water thus consists of more than 33 chemical variants of HOH, but since these variants are present in minute amounts, the properties are dominated by the HOH species.

2.4 ASSOCIATION OF WATER MOLECULES

The V-like shape of an HOH molecule and the polarized nature of the O—H bond result in an asymmetric charge distribution within the molecule and a dipole moment in the vapor state of 1.84 D for pure water. Polarity of this magnitude results in considerable intermolecular attractive forces, and hence water molecules associate with considerable tenacity. Note, however, that the unusually large intermolecular attractive force of water cannot be fully accounted for solely on the basis of the large molecular dipole moment. This is to be expected, since dipole moments are a property of the entire molecule, and give no indication of the degree to which individual charges are exposed or of the geometry of the molecule, aspects that have an important bearing on the intensity of the intermolecular association.

The large intermolecular attractive forces between water molecules can be explained satisfactorily in terms of their ability to engage in multiple hydrogen bonding associations in a three-dimensional manner. As compared with covalent bonds (average bond energy about 335 kJ/mol) hydrogen bonds are weak (typically 2–40 kJ/mol) and have greater and more variable lengths. The oxygen–hydrogen

bond has a dissociation energy of about 11–25 kJ/mol, and ranges in length from around 1.7 to 2.0 Å, as compared to the approximately 1.0 Å length of the oxygen–hydrogen covalent bond [1].

Since electrostatic forces provide a major contribution to the energy of the hydrogen bond, and since an electrostatic model of water is simple and leads to an essentially correct geometric picture of HOH molecules as they are known to exist in ice, further discussion of geometric patterns formed by associating HOH molecules will emphasize electrostatic effects. This simplified approach, while entirely satisfactory for this purpose, will prove to be inadequate, and must be modified if other behavioral characteristics of water, such as the influence of apolar solutes, are to be explained satisfactorily.

The highly electronegative oxygen of the water molecule can be visualized as partially drawing away the single electrons from the two covalently bonded hydrogen atoms, thereby leaving each hydrogen atom with a partial positive charge and a minimal electron shield; that is, each hydrogen atom assumes some of the characteristics of a bare proton. Since the hydrogen–oxygen bonding orbitals are located on two of the axes of an imaginary tetrahedron (Figure 2.1a), these two axes can be considered as representing lines of positive force (hydrogen bond donor sites). Oxygen's two lone pair orbitals can be considered as residing along the remaining two axes of the tetrahedron, representing lines of negative force (hydrogen bond acceptor sites). By virtue of these four lines of force in a tetrahedral orientation, each water molecule has the potential to hydrogen bond with a maximum of four others. The resulting tetrahedral arrangement is depicted in Figure 2.2.

Because each water molecule has an equal number of hydrogen bond donor and acceptor sites, arranged in such a way as to permit three-dimensional hydrogen bonding, it is found that the attractive

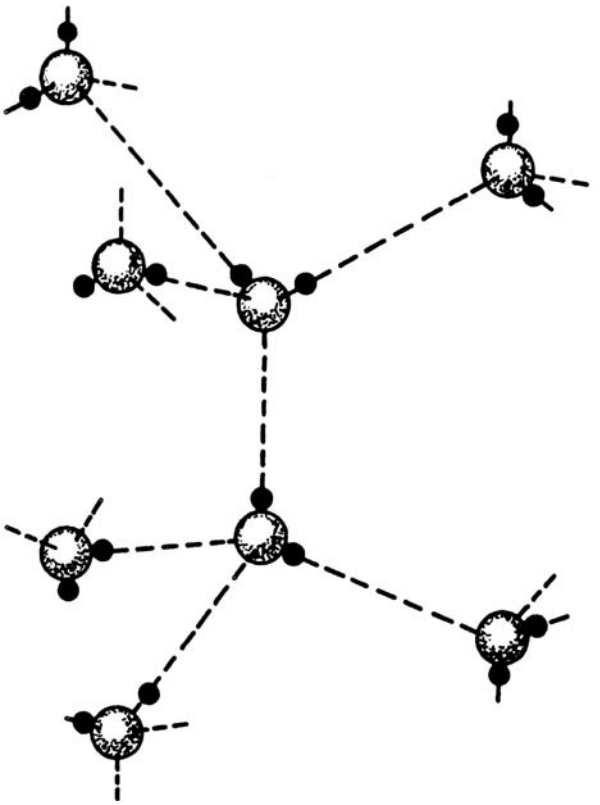

FIGURE 2.2 Hydrogen bonding of water molecules in a tetrahedral configuration. Open circles are oxygen atoms, closed circles are hydrogen atoms. Hydrogen bonds are represented by dashed lines.

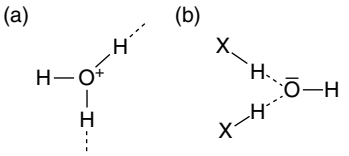

FIGURE 2.3 Structure and hydrogen bond possibilities: (a) for a hydronium ion and (b) for a hydroxyl ion. Dashed lines represent hydrogen bonds, X—H represents a solute or another water molecule.

forces among water molecules are unusually large, even when compared with those existing among other small molecules that also engage in hydrogen bonding associations (e.g., NH_3, HF). Since ammonia (with its tetrahedral arrangement of three donor and one acceptor site) and hydrogen fluoride (with its tetrahedral arrangement of one donor and three acceptor sites) do not have equal numbers of donor and acceptor sites, neither can form three-dimensional hydrogen bonded networks of the type found in water. Both are limited to forming extensive two-dimensional networks, involving fewer hydrogen bonds per molecule than found in water.

Conceptualizing the association of a few water molecules becomes much more complicated when isotopic variants and hydronium and hydroxyl ions are taken into account. The hydronium ion, as a result of its positive charge, would be expected to exhibit a greater hydrogen bond donating potential than nonionized water (Figure 2.3a) and the hydroxyl ion, because of its negative charge, would be expected to exhibit greater hydrogen bond acceptor potential than nonionized water (Figure 2.3b).

This ability of water to engage in extensive three-dimensional hydrogen bonding provides a logical explanation for many of its unusual properties, such as the observed large values of heat capacity, melting point, boiling point, surface tension, and enthalpies of phase transition. All of these can be related to the additional energy necessary to break large numbers of intermolecular hydrogen bonds.

The permittivity (dielectric constant) of water is also influenced by hydrogen bonding. Although water is a dipole, this alone does not account for its large permittivity. It appears that hydrogen-bonded molecular clusters give rise to multimolecular dipoles, effectively increasing the permittivity.

2.5 DISSOCIATION OF WATER MOLECULES

As has already been indicated, two of the species in pure water are the ions produced by the self-dissociation of the molecule, identified in their simplest form as the hydrogen ion, H^+ and the hydroxyl ion OH^-, though in reality these exist in a hydrated form. In pure water, these will exist in equimolar quantities, since they arise from the self-dissociation process.

$$H_2O \longleftrightarrow H^+ + OH^-$$

At 298 K, the equilibrium constant for this dissociation is $K_w = 10^{-14}$ and the pH is 7. It is important to realize that this dissociation is enhanced at higher temperatures, and in consequence, the pH of pure water is temperature dependent. K_w approaches 10^{-12} at 373 K, leading to a pH close to 6 at this temperature. Note that, while a pH of 6 at 298 K implies a concentration of OH^- of 10^{-8} M, at 373 K a pH of 6 implies a concentration of OH^- close to 10^{-6} M.

2.6 STRUCTURES IN PURE WATER SYSTEMS

2.6.1 The Structure of Ice

It is appropriate to discuss the structure of ice before that of liquid water, both because the structure of ice is better understood, and because it is a logical extension of the information presented previously.

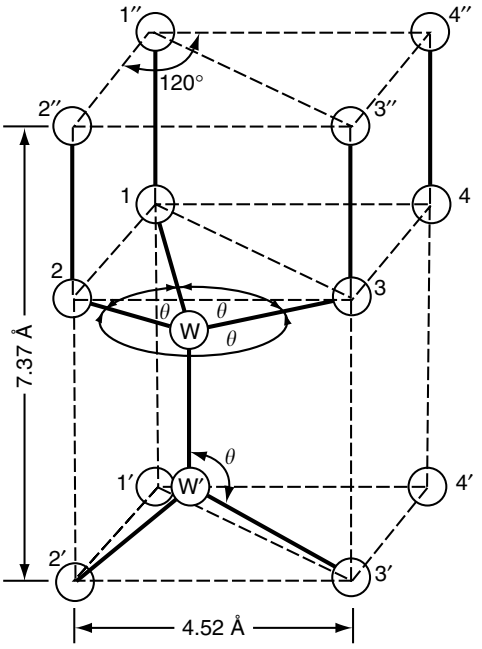

FIGURE 2.4 Unit cell of ordinary ice at 0°C. Circles represent oxygen atoms of water molecules. Nearest neighbor internuclear O—O distance is 2.76 Å. θ is 109°.

Water, with its tetrahedrally directed forces, crystallizes in an open, low density, structure that has been accurately determined. The O—O internuclear nearest neighbor distance, in ice, is 2.76 Å and the O—O—O bond angle is about 109°, very close to the perfect tetrahedral angle of 109.28° (Figure 2.4). The manner in which each HOH bond can associate with four others (coordination number of 4) is readily visualized in the unit cell of Figure 2.4 by considering molecule W and its four nearest neighbors, 1, 2, 3, and W′.

When several unit cells are combined and viewed from the top (down the *c*-axis) the hexagonal symmetry of ice is apparent (Figure 2.5). The tetrahedral substructure is evident from molecule W and its four nearest neighbors, with 1, 2, and 3 being visible and the fourth lying below the plane of the paper, directly under molecule W. When Figure 2.5a is viewed in three dimensions, as in Figure 2.5b, it is evident that two planes of molecules are involved (open and filled circles). These two planes are parallel, very close together, and they move as a unit during the "slip" or flow of ice under pressure, as in a glacier. Pairs of planes of this type comprise the basal planes of ice. By stacking several basal planes an extended structure of ice is obtained. Three basal planes have been combined to form the structure represented in Figure 2.6. Viewed down the *c*-axis, the appearance is exactly the same as that shown in Figure 2.5a indicating that the basal planes are perfectly aligned. Ice is monorefringent in this direction, whereas it is birefringent in all other directions. The *c*-axis is therefore the optical axis of ice. It is interesting to note that, in large sheets of ice, the *c*-axis is often found to be perpendicular to the main plane of the sheet [5]. A fully satisfactory explanation for this has not yet been advanced, though it may reflect the different propagation velocities of ice growth along the different symmetry axes.

With regard to the location of hydrogen atoms in ice, there is general agreement regarding the following:

1. Each line connecting two nearest neighbor oxygen atoms is occupied by one hydrogen atom centered 1 ± 0.01 Å from the oxygen to which it is covalently bonded, and

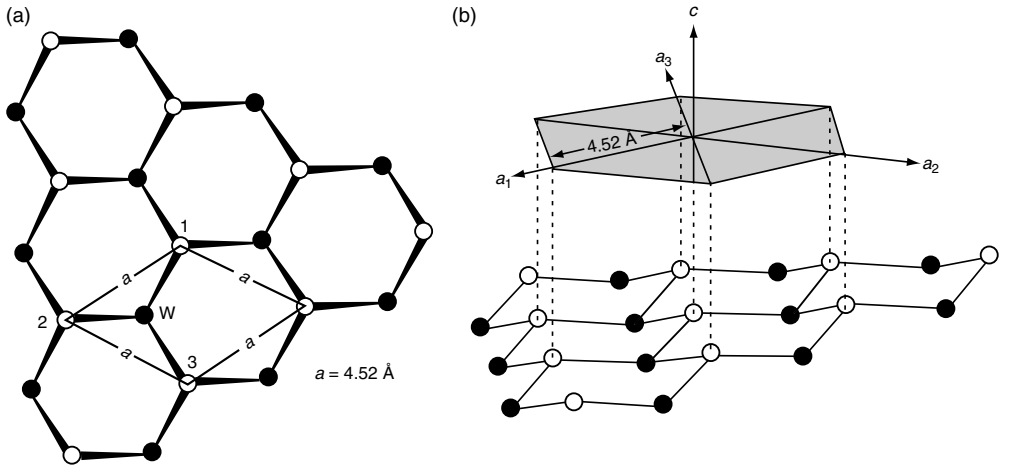

FIGURE 2.5 The basal plane of ice (a combination of two layers of slightly different elevation). Each circle represents the oxygen atom of a water molecule. Open and shaded circles represent, respectively, oxygen atoms in the upper and lower layers of the basal plane. (a) Hexagonal structure viewed down the *c*-axis. Numbered atoms refer to the unit cell of Figure 2.4. (b) Three-dimensional view of the basal plane. The front edge in this view corresponds to the bottom edge of view (a). The crystallographic axes are positioned in accordance with external point symmetry.

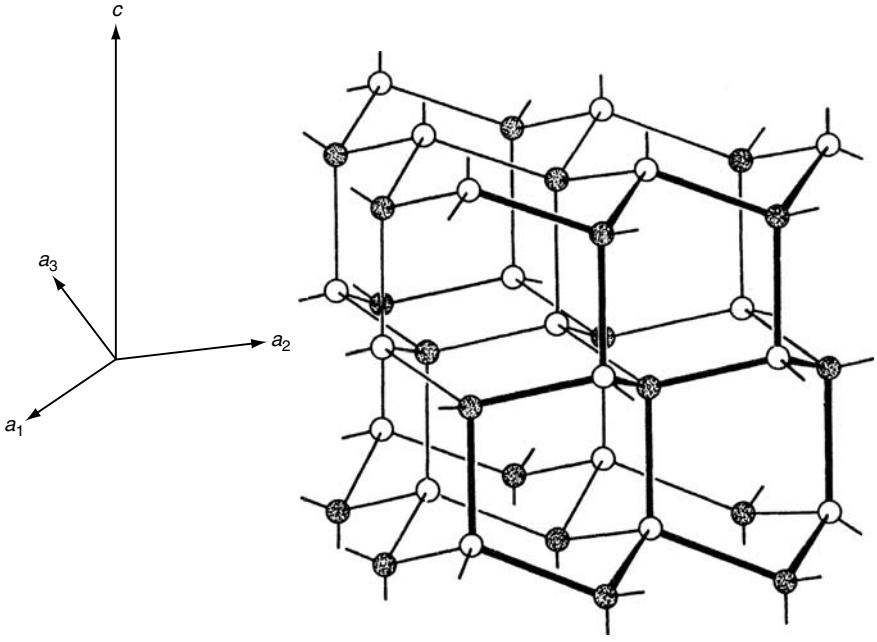

FIGURE 2.6 The extended structure of ordinary ice. Only oxygen atoms are shown. Open and shaded circles represent, respectively, oxygen atoms in upper and lower layers of a basal plane.

1.76 ± 0.01 Å from the oxygen to which it is hydrogen bonded. This configuration is shown in Figure 2.7a.

2. However, if the locations of hydrogen atoms are viewed over time, rather than as a snapshot in time, a somewhat different picture to that described above is obtained. A hydrogen atom

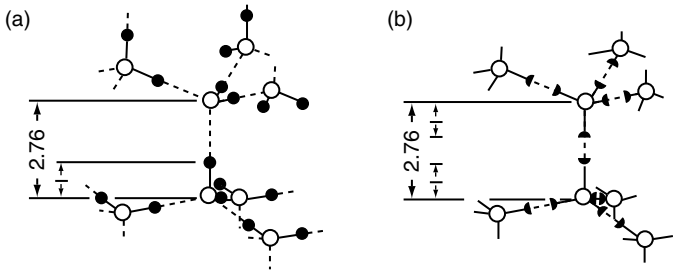

FIGURE 2.7 The location of hydrogen atoms (●) in the structure of ice: (a) instantaneous structure and (b) mean structure (also known as half hydrogen (◖), Pauling, or statistical structure). Open circles are oxygen atoms.

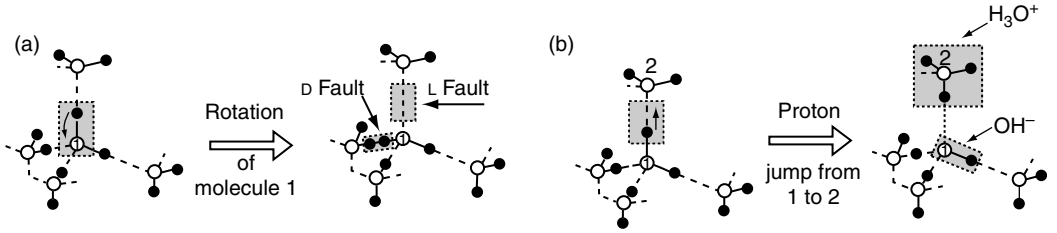

FIGURE 2.8 Schematic representation of proton defects in ice. (a) Formation of orientational defects and (b) formation of ionic defects. Open and filled circles represent, respectively, oxygen and hydrogen atoms. Solid and dashed lines represent, respectively, chemical bonds and hydrogen bonds.

on a line connecting two nearest neighbor oxygen atoms, X and Y, can situate itself in one of two possible positions, either 1 Å from X or 1 Å from Y. Since these two positions have equal probability of occupation, it is believed that each position is occupied on average half of the time. This is possible because, except at extremely low temperatures, HOH molecules can cooperatively rotate, and therefore allow hydrogen atoms to "jump" between adjacent oxygen atoms. A representation of the resulting mean structure, known variously as the half hydrogen, Pauling, or statistical structure, is shown in Figure 2.7b.

From the perspective of crystal symmetry, ordinary ice belongs to the dihexagonal bipyramidal class of the hexagonal system. Ice can also exist in nine other crystalline polymorphic structures and also in an amorphous or vitreous state of uncertain, but largely noncrystalline structure. Of the 11 total structures, only ordinary hexagonal ice is stable under normal pressure at 0°C.

The true structure of ice is not as simple as the foregoing discussion might indicate. First of all, pure ice contains not only ordinary HOH molecules, but also the isotopic and ionic variants of HOH that have been noted as minor constituents of water. Fortunately, we can in most instances ignore the structural influence of the isotopic variants, as they are present in such small amounts. Structurally, major consideration need only be given to the contributions from HOH, H^+ (H_3O^+), and OH^-.

Real ice crystals are never perfect, and the structural defects encountered are usually of the orientational type (caused by proton dislocation accompanied by neutralizing orientational adjustments) or ionic type (caused by proton dislocation with formation of H_3O^+ and OH^-) (see Figure 2.8). The presence of these structural defects provides a means for explaining the unexpectedly high mobility of protons in ice, and also the relatively small decrease in electrical conductivity that occurs when water is frozen, where intuitively one might expect a large loss in conductivity on solidification.

In addition to the atomic mobilities involved in crystal (lattice) defects, there are other types of motional activity in ice. Each HOH molecule in ice is believed to vibrate with a root mean amplitude

of vibration (assuming each molecule vibrates as a unit) of about 0.4 Å at −10°C [5]. Additionally, the individual HOH molecules that presumably occupy some of the interstitial spaces of ice can apparently diffuse slowly through the lattice rather than being trapped in a particular interstitial space.

Ice therefore is far from being a static or homogeneous molecular assembly, and its characteristics are dependent upon temperature. Although HOH molecules in ice are four coordinated at all temperatures, it is necessary to reduce the temperature to about −180°C or lower to constrain the hydrogen atoms to only one of the many possible configurations. Hence, only at temperatures near −180°C or lower will all hydrogen bonds be intact, and as the temperature is raised the mean number of intact (fixed) hydrogen bonds will gradually decrease.

2.6.2 The Structure of Water (Liquid)

At first sight, the concept of structure in a liquid may seem strange since fluidity is the essence of the liquid state, yet it is an old, and well-accepted idea [6] that liquid water possesses some level of structure, not sufficiently established to produce long-range rigidity, but yet far more organized than that of the vapor state, and sufficient in extent to cause the orientation and mobility of any given water molecule to be influenced by neighboring water molecules. One useful conceptual approach has been to think of the structure in the liquid as a series of short-term structured associations, always rapidly interconverting, but nevertheless maintaining an average degree of structure within the liquid at all times.

Evidence for this view of water as a structured liquid is extensive and compelling. For example, water is an "open" liquid, with a density only 60% of that to be expected of a liquid in which the molecules are close packed. Partial retention of the open, hydrogen-bonded tetrahedral arrangement of ice can easily account for the low density of liquid water. Furthermore, while the enthalpy of fusion of ice is unusually high for a solid, it corresponds to the energy that would be required to break only about 15% of the hydrogen bonds believed to exist in ice. Although this does not necessarily imply that 85% of the hydrogen bonds existing in ice are retained in liquid water (e.g., more bonds could be broken but the resulting change in energy could be masked by a simultaneous increase in van der Waals interactions), the results of many separate studies strongly support the concept that many water–water hydrogen bonds continue to exist in the liquid, with the extent of hydrogen bonding decreasing as the temperature of the liquid increases [1,7].

Elucidation of the structure(s) of pure liquid water is an extremely complex and challenging problem. Many theories have been proposed, but all are incomplete, oversimplified, and subject to weaknesses that are quickly cited by proponents of rival theories. This is a healthy situation, which should eventually result in an accurate structural description of liquid water. In recent years, the increased power of computers has rendered feasible computer simulations of the molecular dynamics of water, governed by the equations of motion, and molecular potential functions that seek to approximate the significant interactive modes of the water molecule [8–10]. These simulations, limited as they are by the errors and approximations of the chosen potential function, are found to display many of the characteristic properties of water, and are providing powerful new insights into the realities of liquid water. Visual displays of the movements of the molecules represented in the simulation are very instructive, but difficult to capture on paper. Notwithstanding the increasing sophistication of these simulations, and the valuable insights that they provide, it is a valuable exercise to consider models generated before access to such raw computational power as exists today became commonplace.

Three general types of model for liquid water have been proposed: mixture models, interstitial models, and continuum models (also termed homogeneous or uniformist models) [11,12]. Mixture models embody the concept of intermolecular hydrogen bonds being momentarily concentrated in bulky clusters of water molecules that exist in dynamic equilibrium with more dense species, with "momentarily" indicating a timescale of 10^{-11} s or thereabouts [12]. The molecular dynamic

computer simulations are often an embodiment of this type of approach, with the simulation providing a time sequence of snapshots of the location (and often orientation) of the constituent molecules represented in the model. The exact characteristics exhibited by the model depend upon the interaction potential function ascribed to water, and many different potential functions have been proposed and utilized, each with its particular strengths and weaknesses.

Continuum models involve the idea that intermolecular hydrogen bonds are distributed uniformly through the sample, and that many of the bonds existing in ice simply become distorted rather than broken when ice is melted. It has been suggested that this permits a continuous network of water molecules to exist that is, of course, dynamic in nature, with the distortions able to relocate in space by transfer across the network [13,14].

The interstitial model involves the concept of water retaining, with little distortion, either an ice-like or clathrate-type hydrogen-bonded network structure with unbonded individual water molecules filling the interstitial spaces of the network. In all three models, the dominant structural feature is the concept of a hydrogen-bonded association of liquid water in ephemeral, distorted tetrahedra. All models also permit individual water molecules to frequently alter their bonding arrangements by rapidly terminating one hydrogen bond in exchange for a new one, while still maintaining, at constant temperature, a constant degree of hydrogen bonding and structure for the system as a whole.

In many respects, the more recent computer models demonstrate facets of each of the more traditional models [10]. Evidence is found for changing orientations of hydrogen bonds and for relocation of water molecules in positions not supported by a traditional hydrogen-bonded network. A variety of modeling studies have successfully approximated the observed behaviors of water. In the computer models, which produce time-averaged pictures, while hydrogen bonding is clearly very important, the appearance of well-defined structures, as might be implied by the simpler models, does not occur.

It is now possible to discuss the seemingly anomalous low viscosity of water. This attribute is readily reconcilable with the types of structures that have been described, since the hydrogen-bonded arrangements of water molecules are highly dynamic, allowing individual molecules within a timeframe of nano- to picoseconds to alter their hydrogen-bonding relationships with neighboring molecules, thereby facilitating mobility and fluidity. The unusually high heat capacity of liquid water is seen to be in part a reflection of the energy required to break additional hydrogen bonds as the temperature is increased. The high enthalpy of vaporization reflects the breaking of most or all remaining hydrogen bonds as the liquid vaporizes, since most molecules in the vapor are believed to be monomers.

The degree of intermolecular hydrogen bonding among water molecules is, of course, temperature dependent. Ice at 0°C has a coordination number (number of nearest neighbors) of 4.0, with the nearest neighbor distance being 2.76 Å. With input of the enthalpy of fusion, melting occurs. The enthalpy of fusion reflects some hydrogen bonds being broken (the distance between nearest neighbors increases) and other hydrogen bonds becoming strained as water molecules assume a fluid state with associations that are, on average, more compact. As the temperature is raised, the coordination number increases from 4.0 in ice at 0°C to 4.4 in water at 1.50°C then to 4.9 at 83°C. Simultaneously, the distance between nearest neighbors increases from 2.76 Å in ice at 0°C to 2.9 Å in water at 1.50°C then to 3.05 Å at 83°C [15,16].

It is evident, therefore, that the ice to water transformation is accompanied by an increase in the distance between nearest neighbors (decreased density), and by an increase in the average number of nearest neighbors (increased density) with the latter predominating during the phase change to yield the familiar net increase in density associated with melting. Further warming above the melting point causes the density to pass through a maximum at 3.98°C, then gradually decline. It is apparent that the effect of an increase in coordination number is predominant at temperatures between 0°C and 3.98°C, and that an effect of increasing distance between nearest neighbors (thermal expansion) is predominant above 3.98°C.

2.7 PHASE RELATIONSHIPS OF PURE WATER

Up to this point, we have considered only molecular and structural aspects of water; in other words, interactions and interrelationships at the microscopic and submicroscopic level. It is appropriate now to discuss the observable phase behavior of water, as this will be relevant not just to our appreciation of the properties of pure water, but also to later discussions on the behavior of aqueous solutions under a wide range of conditions of temperature and pressure. When we study the phase relationships of pure water, the influences of both temperature and pressure must be considered.

Figure 2.9a shows the phase diagram for pure water. Of particular relevance to food science is the vapor–liquid equilibrium line, and also the pressure dependence of stable forms of ice. As mentioned earlier, several forms of ice have been identified, each stable in a particular region of the temperature–pressure diagram. Under the conditions of temperature and pressure utilized in food processing, the only ice phase of interest is ice I. It is noteworthy that, for ice I, with increasing pressure, the melting point of ice Ih decreases (Figure 2.9b). Particularly, note that at a pressure of 270 MPa the melting point of ice Ih is below −20°C. This fact finds application in techniques such as pressure shift freezing [17,18] where a food is cooled to −20°C while experiencing a high pressure. Under these conditions, the material is above the freezing point of ice and so water remains liquid, though enthalpy (heat content) is reduced. On release of the pressure, freezing is very rapid, as the stable form of water under the new conditions of temperature and pressure is ice, and the unchanged heat content of the sample is that of a sample containing a significant fraction of ice. A reverse thawing process has also been designed, in which a frozen material is subjected to a sufficiently high pressure to enable water to become the stable phase with no change in temperature. Since pressure can be applied almost instantaneously, thawing is immediate. After increasing the temperature to above 0°C, the applied pressure can be released without ice forming. Thus, a thawing process in which the phase change is uniform through the material can be achieved, rather than being progressive, reflecting the geometric pattern of temperature change controlled by the processes of heat transfer. At higher pressures than those where ice I is the stable phase, other forms of ice exist such as ice II, ice III, ice IV, and so forth. These forms are not found under any conditions relevant to the handling and processing of foods and need not be discussed further.

In considering equilibria in vapor–liquid systems, note that the vapor pressure of pure liquid water increases from 610 Pa at 0°C to 101,323 Pa at 100°C. It is also possible to measure a vapor pressure of water above undercooled liquid water at temperatures below 0°C, under conditions where the equilibrium form of HOH is ice. These pressures are always higher than the equilibrium vapor pressure of water above ice at the same temperature (Table 2.3). The significance of these observations to food science, in particular with respect to the use of relative vapor pressure as an indicator of "water availability" will be discussed later in this chapter. Since water is an important component of food, we need to develop some system(s) to describe the amount, state, and condition of the water in the food. However, before settling upon a suitable descriptor, first we must consider systems more complex than pure water to understand the influence of the molecular environment on the properties exhibited by water at both the molecular and the bulk level.

2.8 WATER IN THE PRESENCE OF SOLUTES

In all food systems, both water and solutes are present. It is therefore necessary to discuss the effects of solutes on the nature and behavior of a collection of water molecules including its solvent properties.

2.8.1 Ice in the Presence of Solutes

The presence of solutes influences both the amount (through thermodynamic effects) and the propagation patterns (through kinetic effects) of ice in an aqueous system. As the concentration

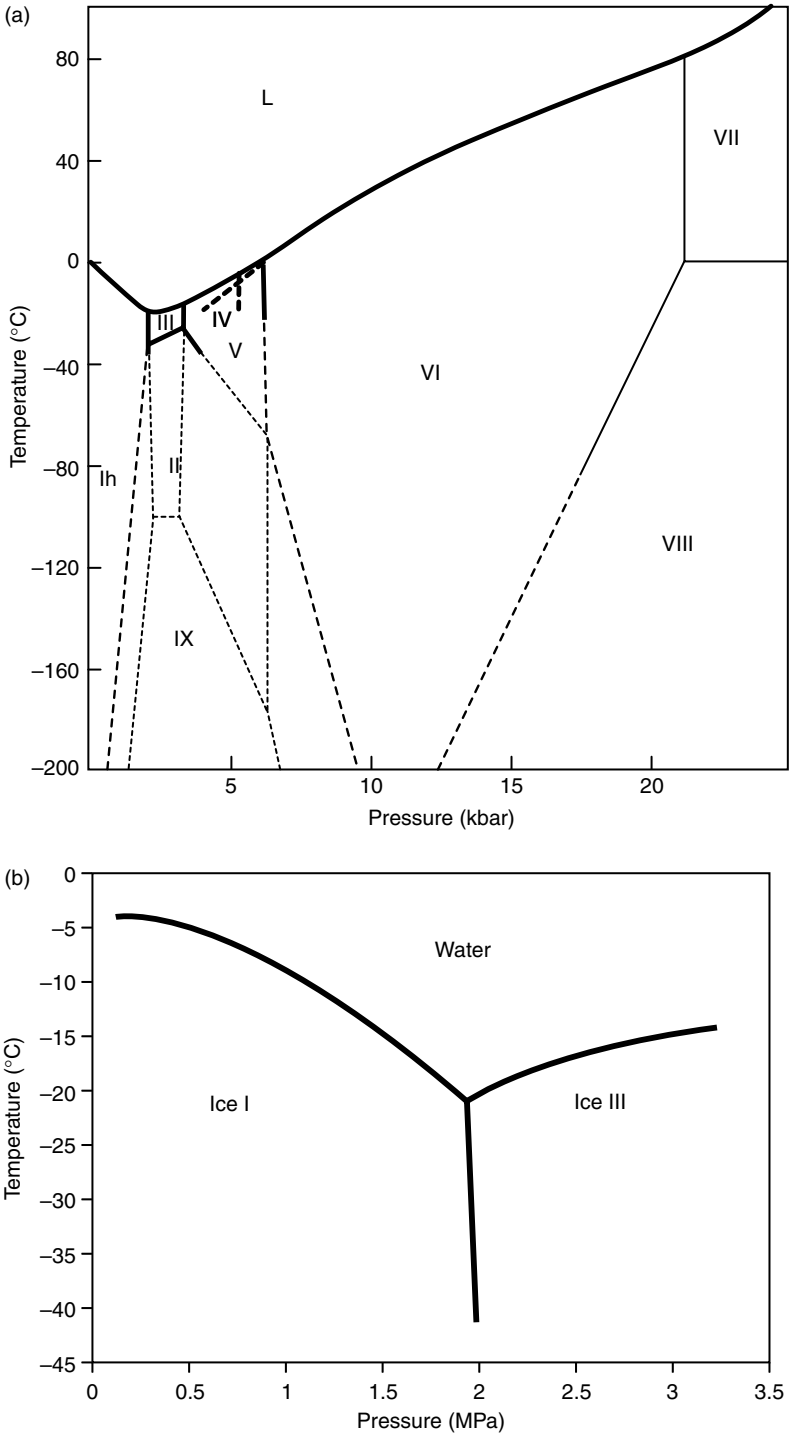

FIGURE 2.9 The pressure–temperature phase diagram for (a) pure water, (b) details of the behavior of liquid water, ice I, and III.

TABLE 2.3
Vapor Pressures and Vapor Pressure Ratios of Water and Ice

Temperature (°C)	$(p^o)_w$ Water (kPa)	$(p^o)_i$ Ice (kPa)	$((p^o)_i/(p^o)_w)$
100	101.325		
90	70.123		
80	47.379		
70	31.181		
60	19.936		
50	12.346		
40	7.382		
30	4.245		
20	2.338		
10	1.228		
0	0.611	0.611	1.00
−5	0.421[a]	0.402	0.954
−10	0.287[a]	0.260	0.905
−15	0.191[a]	0.165	0.863
−20	0.125[a,b]	0.103	0.824
−25	0.0807[a,b]	0.063	0.780
−30	0.0509[a,b]	0.038	0.746
−40	0.0189[a,b]	0.013	0.687
−50	0.0064[a,b]	0.039	0.609

[a] Supercooled liquid.
[b] Calculated data.

Source: Lide, D.R. (Ed.) (1993/1994) *Handbook of Chemistry and Physics*, 74 edn., CRC Press: Boca Raton, FL and Mason, B.J. (1957) *The Physics of Clouds*. Clarendon Press: Oxford, p. 445.

of a particular solute increases, the amount of ice formed at any temperature decreases. This is a manifestation of freezing point depression and colligative effects. Figure 2.10, a simple phase diagram for a binary aqueous system, shows how the freezing point of a binary aqueous solution changes with concentration. Phase and state diagrams will be discussed in more detail later.

The amount and kind of solutes present influence not only the quantity, but also the size, structure, location, and orientation of ice crystals resulting from any particular cooling protocol. Consider, for example, the effects of solute on ice structure. In pioneering studies, Luyet and coworkers [19–21] studied the appearance of ice crystals formed under a range of different cooling conditions in the presence of various solutes including sucrose, glycerol, gelatin, albumin, and myosin. They devised a classification system based on morphology, elements of symmetry, and the cooling velocity required for development of various types of visible ice structures. Their four major classifications of visible ice structures are hexagonal forms, irregular dendrites, coarse spherulites, and evanescent spherulites.

The hexagonal form, which is most highly ordered, is found exclusively in foods, provided that extremely rapid freezing is avoided, and the solute is of a type and concentration that does not interfere unduly with the mobility (ease of spatial reorganization) of water molecules. Gelatin, at high concentrations will, for example, result in more disordered forms of ice crystal. In these early studies, Luyet and coworkers found clear evidence for the existence of a glassy (amorphous) unfrozen phase surrounding the ice crystals at low temperatures and linked the existence of this phase to phenomena such as "collapse" in freeze drying. Around this same time, Rey [22,23] was also performing pioneering studies of the properties of the phases involved in the freeze-drying process and provided similar insights. Though the full significance of these studies was not immediately

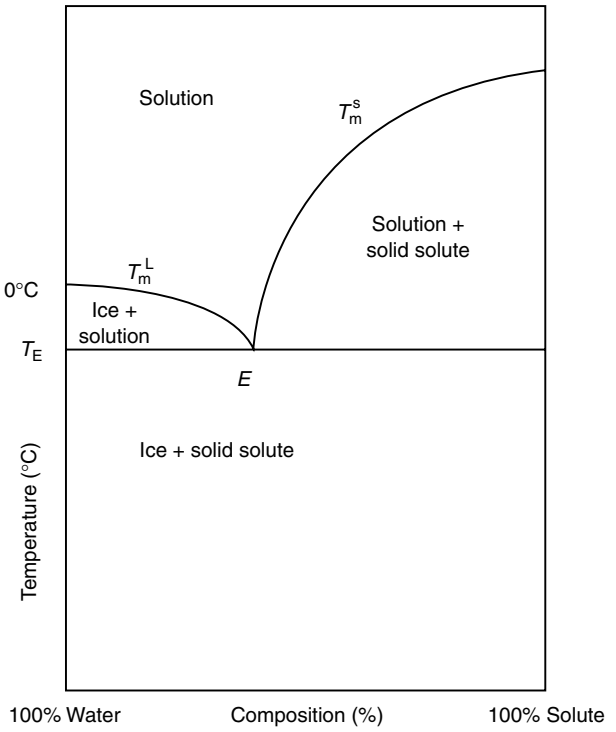

FIGURE 2.10 Schematic binary phase diagram for a simple aqueous system.

realized, they were early precursors to the development of concepts of the role of the glassy state in governing the kinetic properties of frozen systems. These important concepts will be discussed in more detail later.

2.8.2 Water–Solute Interactions in Aqueous Solutions

2.8.2.1 Macroscopic Level

Before dealing with a description of water–solute interactions at a molecular level, it is appropriate to introduce some general observations on the behavior of water. The presence of water has a significant impact on the properties of foods, with properties changing with changes in water content. Terms such as water binding, hydration, water holding capacity have been coined to help describe the influences of water on system properties [24,25]. Often based upon macroscopic observation, these historic terms may prove to be unfortunate when considered in the light of an understanding of the underlying molecular processes that they presumably reflect. Nevertheless, it is important to introduce these descriptive concepts, as they have played an important role in the evolution of our understanding of the properties, and influences, of water in foods.

In the past, "water binding" and "hydration" were often used to describe the general tendency for water to associate with hydrophilic substances, including cellular materials. When used is this context, these terms pertain to the macroscopic level. Although more specialized terms such as "water binding potential" are defined in quantitative terms they still apply only at the macroscopic level. The degree and tenacity of water binding or hydration depend on a number of factors including the nature of the nonaqueous constituent, salt composition, pH, and temperature.

"Water holding capacity" is a term that is frequently employed to describe the ability of a matrix of molecules, usually macromolecules present at low concentration, to physically entrap large amounts

of water in a manner that inhibits exudation under the application of an external, often gravitational, force. Familiar food matrices that entrap water in this way include gels of pectin and starch, and cells of tissues, both plant and animal.

Physically entrapped water does not readily flow from tissue foods, even when they are cut or minced. Nevertheless, this water behaves during processing with properties close to those of pure water. It is easily removed during drying, easily converted into ice during freezing, and is readily available as a solvent. Thus, though its bulk flow is severely restricted, the movement of individual molecules is essentially similar to that of water molecules in a dilute salt solution.

Most of the water in tissues and gels can be categorized as physically entrapped, and impairment of this entrapment capability (water holding capacity) of foods has a profound effect on food quality. Examples of quality defects associated with impairment of water holding capacity are syneresis of gels, thaw exudation from previously frozen foods, and inferior performance of animal tissue in sausage resulting from the decline in muscle pH that accompanies normal physiological events postmortem. In all cases, the quality defect stems from the physical relocation of water molecules in space, but does not necessarily reflect any significant change in the interactive properties of these molecules.

2.8.2.2 Molecular Level: General

The intimate mixing of solutes and water results in alteration of the properties of both constituents as compared to their properties when not mixed. These changes result from molecular interactions, and therefore depend upon the nature of the solute at the molecular level. Ions, or charged groups, interact with water primarily through electrostatic forces. These may enhance, or may interfere with, the normal geometric orientations of water molecules. Predominantly hydrophilic solutes interact strongly with water and cause changes in the structural associations and mobility of adjacent water, and at the same time water changes the reactivity, and sometimes also structure, of the hydrophilic solutes. In contrast, the hydrophobic groups of added solutes interact only weakly with adjacent water, seeming to prefer a nonaqueous environment. However, this weak interaction can have profound structural consequences. The bonding forces existing between water and various kinds of solutes are summarized in Table 2.4.

2.8.2.3 Molecular Level: "Bound Water"

A terminology that is in common use, "bound water" is not an easily defined term and does not refer to a homogeneous entity. It does not necessarily refer even to water truly in some form of bonding association with solute. A unified, coherent descriptive terminology for bound water is difficult,

TABLE 2.4
Classifications of Types of Water–Solute Interactions

Type	Example	Strength	Comments
Water–water	Hydrogen bond	5–25 kJ/mol	
Dipole–ion	Water-free ion	40–600 kJ	Depends on ion size and charge
	Water-charged substituent on organic molecule		Influenced by pH and ionic strength
Dipole–dipole	Water–protein NH	5–25 kJ/mol	
	Water–protein CO	5–25 kJ/mol	
	Water-side chain OH	5–25 kJ/mol	
Hydrophobic hydration	Water + R → R(hyd)	Low	Cumulative sum larger
Hydrophobic interaction	2R(hyd) → R_2(hyd) + H_2O	Low	Cumulative sum larger

as numerous, often conflicting definitions illustrate, and there is no consensus about which one, if any, is the best. The term is controversial, frequently misused, and often poorly understood, and many scientists have suggested that the use of the term be discontinued. Although such a step would indeed be desirable in the interests of more precise communication, the term bound water is so common in the literature that it must be discussed and its severe limitations appreciated.

The following partial list of definitions that have been proposed for bound water illustrates why use of the term has created such confusion [24,25]:

1. Bound water is the equilibrium water content of a sample at some appropriate (and arbitrary) temperature and low humidity.
2. Bound water is that water which does not contribute significantly to permittivity at high frequencies and therefore has its rotational mobility restricted by the substance with which it is associated.
3. Bound water is that which remains unfrozen at some arbitrary low temperature, usually −40°C or lower.
4. Bound water is that which is unavailable as a solvent for additional solutes.
5. Bound water is that which produces line broadening in experiments involving proton nuclear magnetic resonance.
6. Bound water is that which moves with the macromolecule in experiments involving sedimentation rates, viscosity, or diffusion.
7. Bound water is that which exists in the vicinity of solutes and other nonaqueous substances, and has apparent properties differing significantly from those of "bulk" water in the same system.

All of these definitions have some validity under the appropriate conditions, but few will produce the same value when they are separately applied to the same, given system. Also, in some cases, the value obtained for bound water using a particular technique and definition will depend on the total water content of the system even when this content is already in excess of the amount of the bound water determined.

From a conceptual point of view it can be useful to think of bound water as somehow imperfectly describing the "water that exists in the vicinity of solutes and other nonaqueous constituents, and that as a result of its location exhibits apparent properties that are significantly altered from those of 'bulk water' in the same system." Bound water can be thought of as in some way having "hindered mobility" as compared to bulk water, not as being "immobilized." In a typical food of high water content, this type of water comprises only a minute part of the total water present, corresponding approximately to the first layer of water molecules adjacent in space to hydrophilic groups. Bear in mind, however, that this is not a static population of water molecules.

This subject of bound or hindered water will be discussed further in the section dealing with molecular mobilities in frozen systems.

2.8.2.4 Interactions of Water with Ions and Ionic Groups

Individual ions and also the ionic groups of organic molecules appear to hinder or influence the mobility of water molecules to a greater degree than do any other types of solutes. The strength of electrostatic water–ion bonds is greater than that of water–water hydrogen bonds, but still much less than that of covalent bonds.

The accepted normal structure of pure water (based on a hydrogen-bonded, generally tetrahedral arrangement) may be disrupted by the addition of dissociable solutes. Water and simple inorganic ions undergo dipole–ion interactions. The example in Figure 2.11 illustrates hydration of the NaCl ion pair. Only the first-layer water molecules in the plane of the paper, oriented by the radial electrical fields associated with the ions, are depicted. In a dilute solution of the ions in water, a second layer of

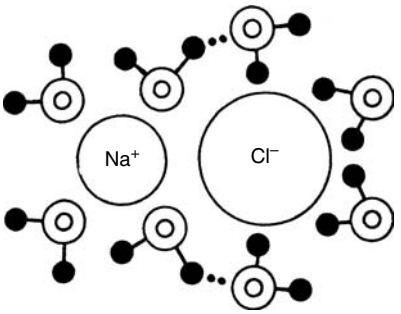

FIGURE 2.11 Likely arrangement of water molecules adjacent to sodium chloride ion pair. Only water molecules in the plane of the paper are represented.

water molecules beyond this first layer is believed to exist in a structurally perturbed state, because of the conflicting structural influences of first-layer water surrounding the charged ions and the more distant, tetrahedrally oriented "bulk-phase" water that is remote from the influence of the radial electrical fields surrounding the ions. In concentrated salt solutions, where individual ion electrical fields would be expected to overlap, bulk-phase water would not exist, and water structure would be dominated by the ions.

There is abundant evidence indicating that some ions in dilute aqueous solution, have a "net structure breaking" effect (solution more fluid than pure water) whereas others have a "net structure forming" effect (solution less fluid than pure water). This is not to suggest that this "structure" is that existing in pure water. The term "net structure" refers to all kinds of structures, either the normal organization of water, or new types of water organization. Clearly, from the standpoint of "normal" water structure, all ions are disruptive, since normal water structure does not possess radial symmetry [26].

The ability of a given ion to alter net structure is related closely to its polarizing power (charge divided by radius) or simply the strength of its electric field. Small and/or multivalent ions (such as Li^+, Na^+, H_3O^+, Ca^{2+}, Ba^{2+}, Mg^{2+}, Al^{3+}, F^-, and OH^-) have strong electric fields and are net structure promoters. The structure imposed by these ions more than compensates from any loss in normal water structure. These ions strongly interact with four to six first-layer water molecules, causing them to be less mobile, and to pack more densely than HOH molecules in pure water. Ions that are large and monovalent, such as K^+, Rb^+, Cs^+, NH_4^+, Cl^-, Br^-, I^-, NO_3^-, BrO_3^-, IO_3^-, and ClO_4^- have rather weak electric fields and are net structure breakers although the effect is very slight with K^+. These ions disrupt the normal structure of water and fail to impose a compensating amount of new structure.

Ions, of course, have important effects in addition to their influence on water structure. Through their varying abilities to hydrate (compete for water), influence the permittivity of the aqueous medium, and govern the thickness of the electrical double layer around colloids, ions profoundly influence the "degree of hospitality" extended to other solutes present in the aqueous medium, and also to substances suspended in the medium. Early recognition of this was the Hofmeister or lyotropic series, which ranked ions in order of their effectiveness in salting-in or salting-out proteins, or their effectiveness in influencing various other properties such as colloid stability. It is encouraging to note that the empirically derived Hofmeister series correlates well with a series based on the expected structural influences of the various ions [27,28].

2.8.2.5 Interaction of Water with Neutral Groups Capable of Hydrogen Bonding (Hydrophilic Solutes)

Interactions between water and nonionic, hydrophilic solutes are weaker than water–ion interactions and about the same strength as those of water–water hydrogen bonds. Depending on the strength of the

water–solute hydrogen bonds, first-layer water (i.e., water immediately adjacent to the hydrophilic species), may or may not exhibit reduced mobility and other altered properties as compared to bulk-phase water.

Solutes capable of hydrogen bonding might at first glance be expected to enhance, or at least to not disrupt the normal structures of pure water. This simplistic expectation ignores the importance of spatial orientation and spatial location to the existence of a viable hydrogen-bonded network. In some instances it is found that the distribution and orientation of the hydrogen-bonding sites of the solute are geometrically incompatible with those existing in pure water. Such solutes frequently have a disruptive influence on the normal three-dimensional, tetrahedrally oriented structure of pure water. Urea is a good example of a small hydrogen-bonding solute that, for geometric reasons may have a marked disruptive effect on the normal structure of water. Conversely, some molecules may have potential hydrogen-bonding hydrophilic groups at orientations and spacings compatible with the hydrogen-bonding structures of water. The simple carbohydrates provide an example. It has been noted that they possess equatorial hydroxyl groups that have similar spatial relationships to those of water molecules in a cluster (see Figure 2.12). Such a degree of compatibility might even enhance the total level of hydrogen bonding. It should be noted that, since the oxygen atom spacings of water and of the individual molecules are temperature dependent, it is not claimed that there is an exact correspondence under all situations, rather it is claimed that there is a close correspondence, and thus potentially a facilitated interaction.

It is important to understand that the total number of hydrogen bonds per mole of water might not be significantly altered by the addition of a hydrogen-bonding solute that disrupts the normal structure of water. This is possible since disrupted water–water hydrogen bonds may be replaced by

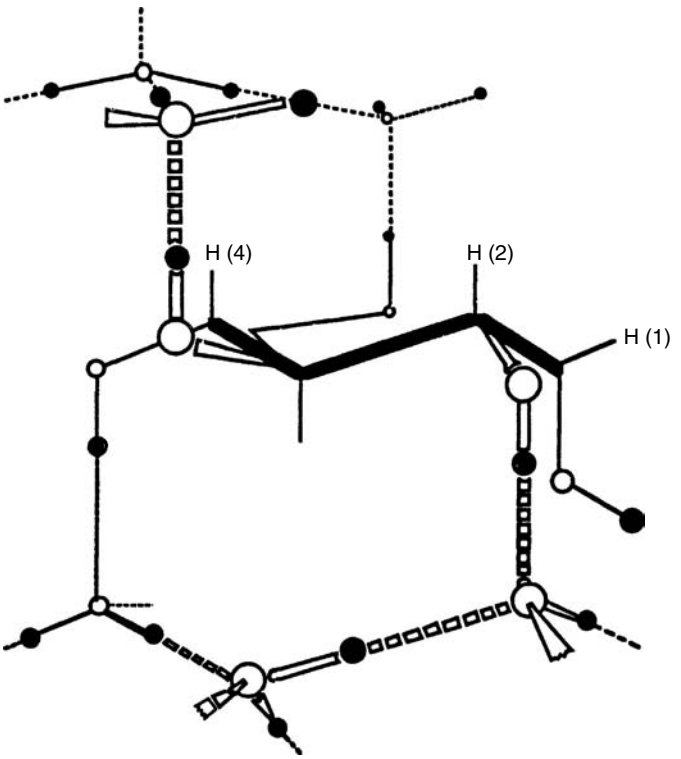

FIGURE 2.12 Possible association of D-glucose with tetrahedrally arranged water molecules. The side view of the pyranose ring is represented by a heavy line. Oxygen and hydrogen atoms of water molecules are represented by open and filled circles, respectively. The hydroxymethyl protons are not shown. (From Suggett, A. (1976) *J. Solution Chem.* **5**: 33–46.)

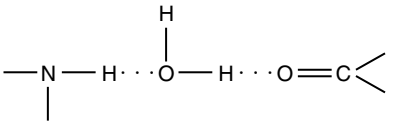

FIGURE 2.13 Hydrogen bonding (dotted lines) of water molecules to two kinds of functional groups commonly occurring in proteins.

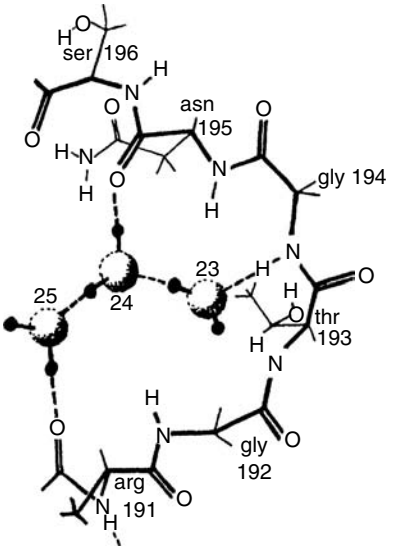

FIGURE 2.14 Example of a three molecule water bridge in papain: 23, 24, and 25 are the water molecules of the bridge. (From Berendsen, H.J.C. (1975) In *Water, a Comprehensive Treatise* (F. Franks, Ed.), Plenum Press: New York, pp. 293–349.)

water–solute hydrogen bonds. Solutes that behave in this manner have little effect on "net structure" as previously defined.

Hydrogen bonding of water can occur with various potentially eligible groups (e.g., hydroxyl, amino, carbonyl, amide, imino groups, etc.). This sometimes results in water bridges, where one water molecule interacts with two eligible hydrogen-bonding sites on one or more solutes. Schematic depiction of hydrogen bonding (dotted lines) of water to two kinds of functional groups found in proteins is shown in Figure 2.13. A more elaborate example involving a three HOH bridge between backbone peptide units is shown in Figure 2.14.

As has been observed for some sugars [29], hydrophilic groups in many crystalline macromolecules are separated by distances very similar to the nearest neighbor oxygen spacing in pure water. Should this spacing prevail in the hydrated macromolecule, this would tend to encourage cooperative hydrogen bonding involving both first- and second-layer water by enhancing the stability (lifetime) of the cluster.

2.8.2.6 Interaction of Water with Nonpolar Substances

The mixing of water and hydrophobic substances such as hydrocarbons, rare gases, and the apolar groups of fatty acids, amino acids, and proteins is, not surprisingly, a thermodynamically unfavorable event ($\Delta G > 0$). However, the free energy of this process is positive not because ΔH is positive, which is typically the case for solutes with a low solubility, but rather because $T\Delta S$ is negative [30].

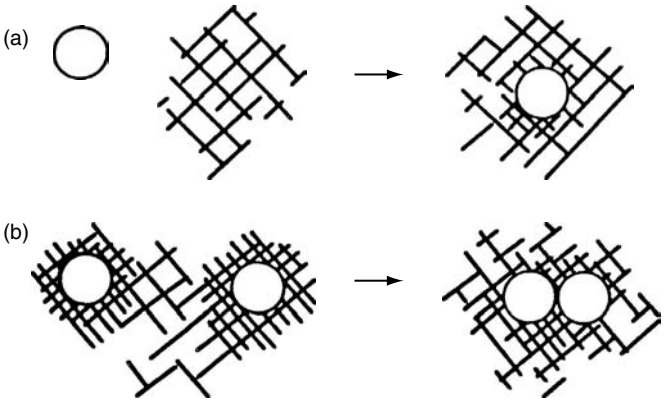

FIGURE 2.15 Schematic depiction of (a) hydrophobic hydration and (b) hydrophobic association. Open circles are hydrophobic groups. Hatched areas are water. (Adapted from Franks, F. (1975) In *Water, a Comprehensive Treatise* (F. Franks, Ed.), Plenum Press: New York, pp. 1–94.)

This reduction in entropy, considered an indicator of increased "order," is thought to occur because of special structures in water that form in the vicinity of these incompatible apolar entities. This process has been termed "hydrophobic hydration" (Table 2.4 and Figure 2.15a).

Because hydrophobic hydration is thermodynamically unfavorable, it follows that the system adjusts in an attempt to minimize the association of water with the apolar entities that are present. Thus, if two separated apolar groups are present, their incompatibility with the aqueous environment serves to encourage their association, thereby lessening the water–apolar interfacial area, a process that is thermodynamically favorable ($\Delta G < 0$). This process, a partial reversal of hydrophobic hydration, is referred to as "hydrophobic interaction" [31] and, in its simplest form, can be represented as

$$\text{R (hydrated)} + \text{R (hydrated)} \rightarrow \text{R}_2\text{ (hydrated)} + \text{H}_2\text{O}$$

where R is an apolar group (Table 2.4 and Figure 2.15b).

Because water and apolar groups exist in an antagonistic relationship, water structure adjusts to minimize contact with apolar groups. The type of water structure that was believed to exist in the layer next to apolar groups is depicted in Figure 2.16. Two aspects of this antagonistic relationship are worthy of further discussion: formation of clathrate hydrates and association of water with hydrophobic groups in proteins.

2.8.2.6.1 Clathrate Hydrates

A clathrate hydrate is an ice-like inclusion compound wherein water, the host substance, forms a hydrogen-bonded cage-like structure that physically entraps a small apolar molecule known as the guest molecule. These compounds are of interest because they represent the most extreme structure forming response of water to an apolar substance, and because microstructures of similar type may occur naturally in biological matter. Clathrate hydrates can be crystalline. Clathrate crystals can easily be grown to visible size, and some are stable at temperatures above 0°C, provided the pressure is sufficient [32]. The guest molecules of clathrate hydrates are characteristically low molecular weight compounds with sizes and shapes compatible with the dimensions of host water cages comprised of 20–74 water molecules. Typical guests include low molecular weight hydrocarbons and halogenated hydrocarbons; rare gases; short chain primary, secondary, and tertiary amines; and alkyl ammonium, sulfonium, and phosphonium salts. Direct interaction between water and guest is slight, usually involving nothing more than weak van der Waals forces; indeed often the guest molecule is free to

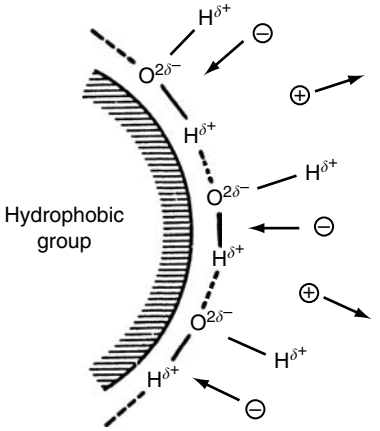

FIGURE 2.16 Proposed water orientation at a hydrophobic surface. (Adapted from Lewin, S. (1974) *Displacement of Water and its Control of Biochemical Reactions*. Academic Press: London.)

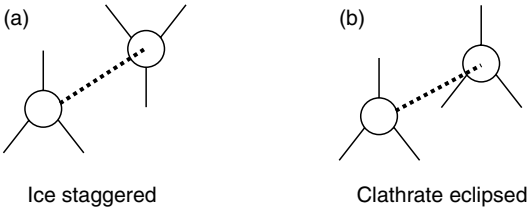

FIGURE 2.17 Relative orientation of hydrogen-bonded water molecules: (a) staggered conformation (ice) and (b) eclipsed conformation (clathrate).

rotate within the cavity. Clathrate hydrates are the extraordinary result of water's attempt to minimize contact with hydrophobic groups. While at first sight the water structure in a clathrate hydrate is very different from the structure in ice, this structure arises from a subtle change in the geometry of the hydrogen bond. In ice, water molecules, tetrahedrally coordinated to their neighbors, have their hydrogen bonds in the staggered conformation if one looks along the direction of an oxygen–oxygen link while in a clathrate hydrate the geometry of the tetrahedral coordination of the water molecules is in the eclipsed form (Figure 2.17). This rotation of 60° in the bond orientation results in structures in which three of the four hydrogen bonds of a water molecule can help form the curved surface (like a geodesic surface) of a cage, while the fourth hydrogen bond projects out normal to this surface. Thus, there are no hydrogen bonds projecting into the internal cavity, and hence no unfavorable interaction with any apolar group within the cavity. As noted, a small, apolar molecule in the cavity may freely rotate. It is also important to note that, if the free energies of the ice structure and that of the clathrate cage structure (any guest molecules in the cages being absent) are computed, the ice structure is more stable than that of the empty clathrate by only a small amount. Thus, the presence of a suitable guest, stabilizing the cavity through steric interactions, can result in a crystalline structure of much higher stability than ice [33,34].

There is evidence that hydrogen-bonded structures similar to the crystalline clathrate hydrates, but that are less spatially extensive (i.e., hydrogen-bonded multimers with eclipsed orientation), may exist naturally in biological matter [35–37], and should this be the case, these localized structures would be of far greater importance in food science than the crystalline hydrates, since they would be likely to influence the conformation, reactivity, and stability of molecules such as proteins. For example, it has been suggested that partial clathrate structures may exist around exposed hydrophobic

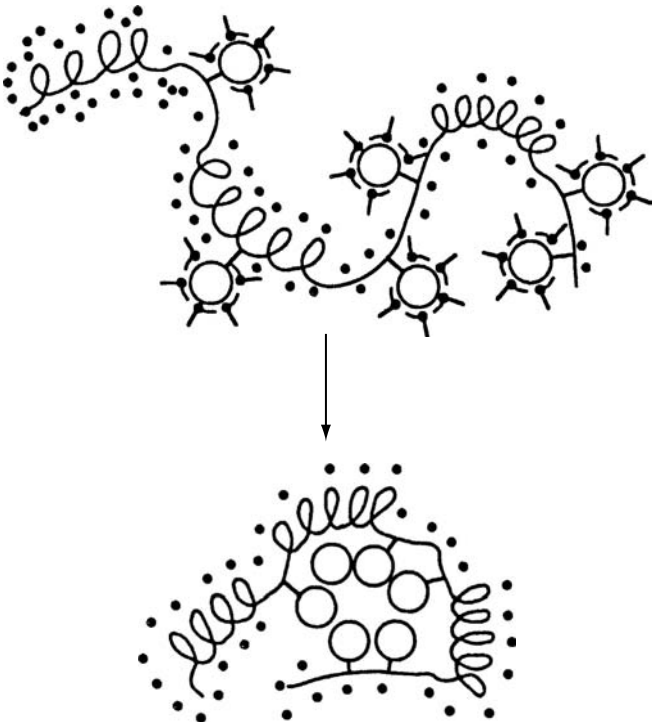

FIGURE 2.18 Schematic illustration of a globular protein undergoing hydrophobic interaction. Open circles are hydrophobic groups. L-shaped entities are water molecules oriented in accordance with proximity to a hydrophobic surface and dots represent water molecules associated with polar groups.

groups of proteins [38]. Figures 2.16 and 2.18 illustrate this concept. It is also possible that clathrate-like structures of water have a role in the anesthetic action of rare gases such as xenon. For further information on clathrate hydrates, the reader is referred to Davidson [32].

Molecular dynamic simulations of aqueous systems with included nonpolar species provide additional evidence for the possible reorientation of the water–water hydrogen bond into a "clathrate-type" orientation as a response to the presence of the nonpolar entity. While in detail, the results of molecular modeling do not show structures with hydrogen-bonding geometries akin to the clathrate-type orientations, the shift in average direction of bonds is consistent with the more simplistic pictorial model. In models incorporating hydrophobic solutes, there is a tendency for hydrogen bonds to be tangential to molecular surfaces [7].

2.8.2.6.2 Interaction of Water with Complex Molecular Structures

Although determination of the arrangement of water molecules near organic molecules is experimentally difficult, this is an active field of research, and useful data have been obtained. The hydrated pyranose sugar ring is shown in Figure 2.12 and a computer simulated cross section of myoglobin [39] is shown in Figure 2.19. Assuming a separation distance of 2.8 Å between hydration sites, and full occupancy of those sites, about 360 HOH molecules would be in the primary hydration shell of myoglobin.

Given the coexistence of polar, hydrophilic, and hydrophobic regions within a large molecule, unavoidable interactions and interferences are inevitable. In proteins, for example, unavoidable association of water with certain hydrophobic groups has an important influence on protein functionality [11,30,38]. The extent of these unavoidable contacts is potentially quite large because

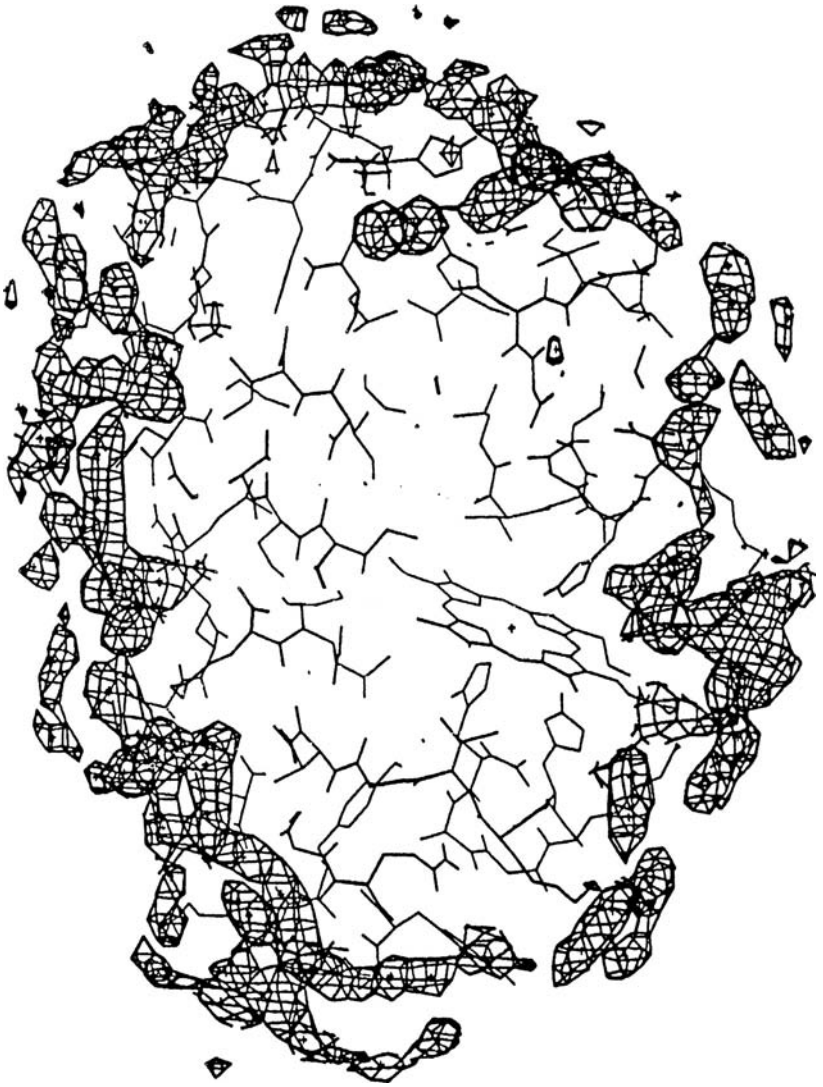

FIGURE 2.19 Cross section of a hydrated myoglobin molecule as determined by a molecular dynamics simulation. Mesh cages represent high probability sites of first layer water molecules. The stick figure represents the protein time-averaged structure. (From Lounnas, V. and B.M. Pettitt (1994) *Proteins: Struc. Func. Genet.* **18**: 133–147.)

apolar side chains exist on about 40% of the amino acids in typical oligomeric food proteins. These nonpolar groups include the methyl group of alanine, the benzyl group of phenylalanine, the isopropyl group of valine, the mercaptomethyl group of cysteine, and the secondary butyl and isobutyl groups of the leucines. The apolar groups of other compounds such as alcohols, fatty acids, and free amino acids also can participate in hydrophobic interactions, but the consequences of these interactions are less important than those involving proteins.

Because the exposure of protein apolar groups to water is thermodynamically unfavorable, the association of hydrophobic groups or hydrophobic interaction is facilitated. This is the process depicted schematically in Figure 2.18. The hydrophobic interaction is believed to be a major driving force for protein folding, causing many hydrophobic residues to assume locations buried in the protein interior. Nevertheless, despite such hydrophobic interactions, it is estimated that nonpolar

groups in globular proteins still typically occupy about 40–50% of the surface area. As a consequence of these surface-located hydrophobic groups, hydrophobic interactions are also regarded as being of primary importance in maintaining the tertiary structure (subunit associations, etc.) of most proteins [40,41]. It is therefore of considerable importance to the structural complexities of proteins that a reduction in temperature causes hydrophobic interactions to become weaker, and hydrogen bonds to become stronger.

Recent applications of molecular modeling to the solvation effects of solutes tend to confirm that the hydrogen-bonding associations of water are important, and indicate that the primary effects of solutes is to modulate the hydrogen-bonding associations that occur within the pure solvent, in particular, causing changes that mirror the changes that are induced in the pure solvent by changes in temperature and pressure, and which are reflected in the equation of state of the solvent water [42].

The foregoing outline of the properties of water, and of aqueous solutions, provides a good foundation for understanding the many roles of water in food systems, and also the influence of the amount and the characteristics of water on the chemistry and microbiology of the food. In the ensuing discussion we will examine the utility of different approaches to understanding the detailed role of water in determining food properties and food stability.

2.9 WATER ACTIVITY AND RELATIVE VAPOR PRESSURE

2.9.1 Introduction

It has long been recognized that a relationship, though imperfect, exists between the water content of food and its perishability, with origins dating back to prehistory. Concentration and dehydration processes are conducted primarily for the purpose of decreasing the water content of a food, simultaneously increasing the concentration of solutes and thereby decreasing perishability.

However, it has also been observed that various types of food with the same water content differ significantly in perishability. Thus, it is evident that water content alone is not a reliable indicator of perishability. This situation is attributable, in part, to differences in the intensity with which water is associated with nonaqueous constituents—one might expect that water engaged in strong associations would be less likely to be able to support degradative activities such as growth of microorganisms and hydrolytic chemical reactions, than would weakly associated water. The term "water activity" (a_w) was developed to reflect the intensity with which water associates with various nonaqueous constituents.

Experience shows that food stability, safety, and other properties can be predicted far more reliably from a_w than from water content. Even so, a_w is not a totally reliable predictor. The reasons for this will be explained in a later section. Despite this lack of perfection, a_w correlates sufficiently well with rates of microbial growth and rates of many degradative reactions to make it a useful indicator of potential product stability and microbial safety. The fact that a_w is specified in some U.S. federal regulations dealing with good manufacturing practices for food attests to its usefulness and credibility [43], and also dictates that this topic be fully explored.

2.9.2 Definition and Measurement

As described in most physical chemistry textbooks, Lewis and Randall rigorously derived the notion of substance "activity" from the laws of thermodynamics, and Scott [44,45] pioneered its application to foods. It is sufficient here to state that,

$$a_w = (f/f^o)_T \tag{2.1}$$

where f is the fugacity of the solvent (fugacity being the escaping tendency of a solvent from solution) and f^o is the fugacity of the pure solvent in some defined standard state. The subscript T refers to the measurement being at constant temperature. At low pressures (e.g., ambient) the difference between f/f^o and p/p^o is less than 1%, so defining a_w in terms of p/p^o is clearly justifiable. Thus,

$$a_w = (p/p^o)_T \tag{2.2}$$

It is important to be aware that this equality is based on the assumption of thermodynamic equilibrium. Since with foods this assumption is generally violated, Equation 2.2 must be considered approximate, and the proper expression is

$$a_w \approx (p/p^o)_T \tag{2.3}$$

In food science, because p/p^o is a readily measured term, which sometimes does not equate to a_w, it is more appropriate to use the term $(p/p^o)_T$ rather than a_w. This practice will be followed here. Relative vapor pressure (RVP) is the name for $(p/p^o)_T$ and these terms will be used interchangeably. Despite the scientific soundness of using RVP rather than a_w, in that RVP does not imply equilibrium, the reader should be aware that the term a_w is in widespread use, appears in other chapters of this book, and is not improper provided the user understands its true meaning and the constraints implied by its use.

Failure of the a_w–RVP approach to be a perfect estimator of food stability occurs for two basic reasons, both violation of the assumptions underlying Equation 2.2 and solute-specific effects. Violation of the assumptions of Equation 2.2 can, but fortunately usually does not, detract unduly from the usefulness of RVP as a technological tool. An exception occurs if dry products are prepared by absorption of water rather than desorption (hysteresis effect). This will be discussed later. Violation of the assumptions of Equation 2.2 does, however, invalidate the use of RVP as a tool for mechanistic interpretation in instances where the theoretical models are based on the validity of these assumptions. This is often true of models of moisture sorption isotherms, where great care should be exercised in applying the apparent conclusions.

In a few instances that can be of great importance, solute-specific effects can cause RVP to be a poor indicator of food stability and safety. This can occur even when the assumptions underlying Equation 2.2 are fully met. In such situations, foods with the same RVP but different solute compositions will exhibit different stabilities and other properties. This is a very important point that must never be overlooked by anyone relying on RVP as a tool for judging the safety and stability of food. Figure 2.20 serves to reinforce this point. These data clearly indicate that the minimum $(p/p^o)_T$ for growth of *Staphylococcus aureus* is dependent upon solute type [46].

Relative vapor pressure is related to percent equilibrium relative humidity (%ERH) of the product environment as follows:

$$\text{RVP} = (p/p^o)_T = \%\text{ERH}/100 \tag{2.4}$$

Two aspects of this relationship are noteworthy. First, RVP is an intrinsic property of the sample, whereas %ERH is a property of the atmosphere established in steady state with the sample. Note that the existence of a steady state does not necessarily assure existence of equilibrium. Second, the equality described in Equation 2.4 exists only if equilibrium has been established between the product and its environment. Establishment of equilibrium is a time-consuming process even with very small samples (<1 g) and almost impossible for large samples, especially at temperatures below 20°C.

Since ERH implies equilibrium, it might therefore be more appropriate to use a modified form of this relationship, which more correctly states that the measured RVP is related to the steady-state

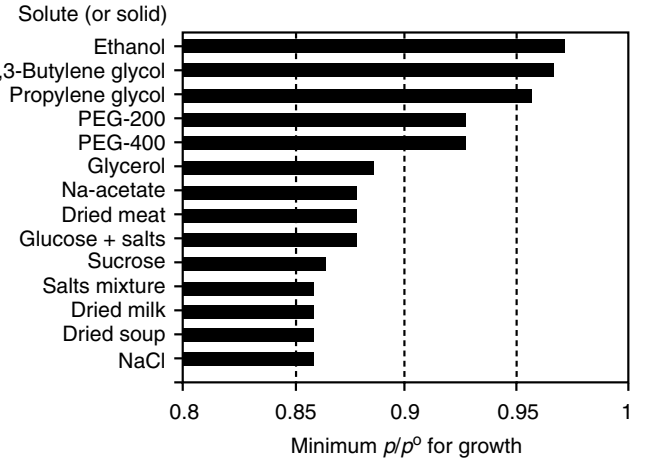

FIGURE 2.20 Minimum RVP for growth of *Staphyloccus aureus* as influenced by solute used to control the RVP. Temperature is close to optimal for growth. PEG is polyethylene glycol. (From Chirife, J. (1994) *J. Food Eng.* **22**: 409–419.)

relative humidity (SSRH) of the product environment:

$$\text{RVP} = (p/p^{\text{o}})_{\text{T}} \approx \%\text{SSRH}/100 \tag{2.5}$$

The RVP of a small sample can be determined by placing it in a closed chamber for a time sufficient to achieve apparent equilibrium (constant weight) and then measuring either pressure or relative humidity in the chamber [47–50]. Various types of instruments are available for measuring pressure (manometers) and relative humidity (electric hygrometers, dew point instruments) [51]. Knowledge of the freezing point depression can also be used to determine RVP, though this relates exactly only to the temperature at the freezing point. On the basis of many collaborative studies, the precision of a_{w} determinations is around ± 0.005 [48].

If one desires to adjust a small sample to a specific RVP, this can be done by placing it in a closed chamber at constant temperature, maintaining sample atmosphere at a constant and known relative humidity by means of an appropriate saturated salt solution or other equivalent method, and storing until constant sample weight is achieved.

2.9.3 Temperature Dependence

Relative vapor pressure is temperature dependent, and the Clausius–Clapeyron equation in modified form provides a means for estimating this temperature dependence. This equation, though based on a_{w}, can often be applied to RVP and takes the form

$$\frac{\text{d} \ln a_{\text{w}}}{\text{d}(1/T)} = \frac{-\Delta H}{R} \tag{2.6}$$

where T is absolute temperature, R is the gas constant, and $-\Delta H$ is the isosteric heat of sorption at the water content of the sample. By rearrangement, this equation can be made to conform to the generalized equation for a straight line. It is then evident that a plot of $\ln a_{\text{w}}$ vs. $1/T$ (at constant water content) should be linear, and if the same holds true for $\ln(p/p^{\text{o}})_{\text{T}}$ vs. $1/T$, then the equation can be used to estimate effective sorption heats for the purposes of comparison. These relationships assume that equilibrium has been achieved, which is often not the case.

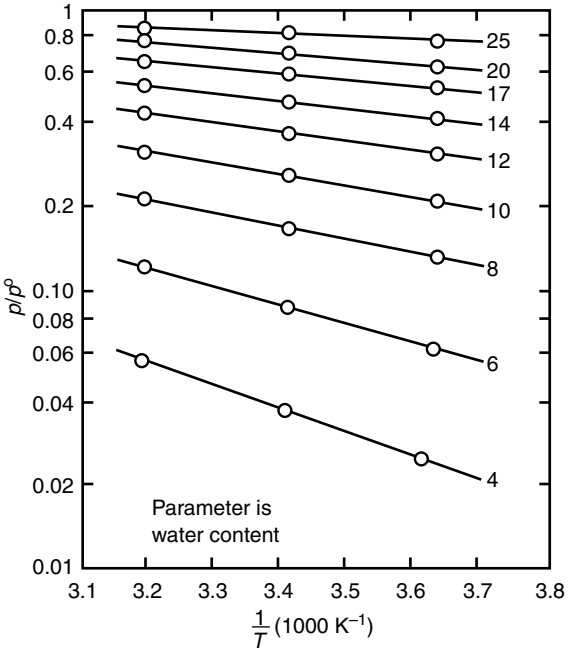

FIGURE 2.21 Relationship between RVP and temperature for native starch of different water contents. Water content values displayed after each line are expressed as g HOH/g dry starch. (From van den Berg, C. and H.A. Leniger (1978) In *Miscellaneous Papers*. Wageningen Agricultural University.)

Linear plots of $\ln(p/p^o)_T$ vs. $1/T$ for native starch at various moisture contents are shown in Figure 2.21. It is apparent that the degree of temperature dependence is a function of moisture content. At a starting $(p/p^o)_T$ of 0.5, the temperature coefficient is 0.0034 K^{-1}over the temperature range 275–313 K (2–40°C). On the basis of the work of several investigators [48,52], temperature coefficients for $(p/p^o)_T$ (temperature range 5–50°C at a starting $(p/p^o)_T$ of 0.5) range from 0.003 to 0.02 K^{-1} for high carbohydrate or high protein foods. Thus, depending on the product, a 10 K change in temperature can cause a 0.03–0.2 change in $(p/p^o)_T$. This behavior can be important for a packaged food because it will undergo a change in RVP with a change in temperature, causing the temperature dependence of its stability to be greater than that of the same product unpackaged.

Plots of $(p/p^o)_T$ vs. $1/T$ are not always linear over broad temperature ranges. For example, they generally exhibit sharp breaks at the onset of ice formation. Before interpreting data at subfreezing temperatures, it is appropriate to consider the definition of RVP as it applies to subfreezing temperatures. As pointed out earlier, undercooled water can exist, as metastable, at temperatures below 0°C. Hence a question arises as to whether the denominator term p^o, the vapor pressure of the pure solvent, should be equated to the vapor pressure of undercooled water, or to the vapor pressure of ice. While pure ice is the equilibrium form of pure water at these temperatures, for useful comparisons to systems above the freezing point, it emerges that the more appropriate choice for standard state is the vapor pressure of undercooled water (1) because the values of RVP at subfreezing temperatures can then, and only then, be directly compared with those at above-freezing temperatures and (2) because making the choice of the vapor pressure of ice as defining p^o would result, for samples containing ice, in a situation whereby the RVP would be unity at all subfreezing temperatures. This second point results because, as is evident from standard thermodynamic relationships, the partial pressure of water of any frozen food is equal to the vapor pressure of ice at the same temperature.

Because the vapor pressure of undercooled water has been measured down to −15°C, and estimated by extrapolation to −30°C (Table 2.3) and the vapor pressure of ice has been measured

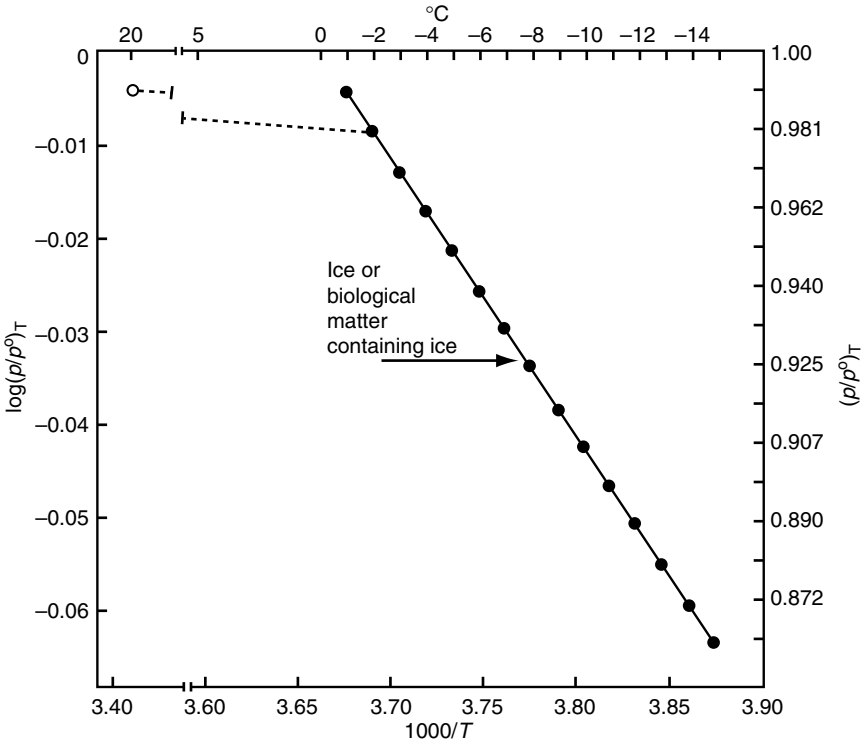

FIGURE 2.22 Relationship between RVP and temperature for aqueous systems above and below freezing. (Modified from Fennema, O. (1978) In *Dry Biological Systems* (J.H. Crowe and J.H. Clegg, Eds.), Academic Press: New York, pp. 297–322.)

to much lower temperatures, it is simple to calculate RVP values for frozen foods based on the undercooled water standard state,

$$a_w = \left[\frac{p_{ff}}{p^o(\text{UCW})}\right]_T = \left[\frac{p_{ice}}{p^o(\text{UCW})}\right]_T \tag{2.7}$$

where p_{ff} is the partial pressure of water in partially frozen food, p^o(UCW) is the vapor pressure of pure undercooled water, and p_{ice} is the vapor pressure of pure ice, all at the same temperature T.

Presented in Table 2.3 are RVP values calculated from the vapor pressures of ice and undercooled water. These values are identical to those of frozen foods at these same temperatures. Figure 2.22 is a plot of $\log(p/p^o)_T$ vs. $1/T$ for a typical aqueous solution, illustrating that (1) the relationship is linear at subfreezing temperatures, (2) the influence of temperature on RVP is typically far greater at subfreezing temperatures than at above-freezing temperatures, and (3) a sharp break occurs in the plot at the freezing point of the sample. Similar behavior is to be expected of biological systems.

Two important distinctions should be noted when comparing RVP values at above- and below-freezing temperatures. First, at above-freezing temperatures, RVP is a function of sample composition and temperature, with the former factor predominating. At subfreezing temperatures, RVP becomes independent of sample composition, and depends solely on temperature since in the presence of an ice phase, RVP values are not influenced by the kind or ratio of solutes present [53]. As a consequence, any subfreezing event that is influenced by the kind of solute present (e.g., diffusion-controlled processes, catalyzed reactions, and reactions affected by the presence or absence of cryo-protective agents, antimicrobial agents, and/or chemicals that alter pH and oxidation–reduction

potential) *cannot* be accurately forecasted based on the RVP value. Consequently, RVP values at subfreezing temperatures are far less valuable indicators of physical and chemical events than are RVP values at above-freezing temperatures. It follows that knowledge of RVP at a subfreezing temperature cannot be used to predict RVP at an above-freezing temperature. Note also that using freezing point depression to estimate a_w or RVP is actually a determination of the break point in the curve.

Second, as the temperature is changed sufficiently to form or to melt ice, the meaning of RVP, in terms of food stability, also changes. For example, in a product at $-15°C$ ($(p/p^o)_T = 0.86$) microorganisms will not grow, and chemical reactions will occur slowly. However, at $+20°C$ and $(p/p^o)_T = 0.86$, some chemical reactions will occur rapidly, and some microorganisms will grow at moderate rates.

This lack of utility of RVP as an indicator of product stability at subfreezing temperatures served as one of the drivers in the development of the molecular mobility approach to understanding food stability relationships, which we shall now discuss. Discussion of moisture sorption isotherms that depict the relationship between moisture content and sample RVP and relate these to the stability of foods is better held until after a discussion of molecular mobility, since the concepts developed in the molecular mobility approach can help clarify some of the relationships between moisture sorption isotherms and product stability.

2.10 MOLECULAR MOBILITY AND FOOD STABILITY

2.10.1 Introduction

Even though the RVP approach has served the food industry well, this should not preclude consideration of other approaches that can supplement or partially replace RVP as a tool for predicting and controlling food stability and processability. Compelling evidence has accumulated to indicate that molecular mobility (Mm: translational or rotational motion) is an attribute of foods that deserves careful attention because it is related causally to many important diffusion-limited properties of food.

In the molecular mobility approach, attention is paid to the mobilities of the constituent molecules. Both rotational and translational mobilities are considered relevant. This consideration of mobilities implies that careful attention should be given to the diffusional aspects of many reactions, and in particular, the importance of diffusion-limited reactions to the quality in many foods.

2.10.2 The Early History

As indicated earlier, Luyet and associates in the United States [19,20] and Rey and associates in France [22,23] were the first to draw attention to the relevance of Mm (glassy states, recrystallization, collapse temperatures during freeze drying) to the properties of biological materials. Many of the basic concepts pertaining to Mm in nonequilibrium systems consisting of synthetic amorphous polymers were formulated by Ferry and associates [54,55] and White and Cakebread [56,57] described the important role of glassy and supersaturated states in various sugar containing foods, and suggested that the existence of these states had an important influence on the stability and processability of many foods. Duckworth et al. [58] demonstrated the relevance of Mm to rates of nonenzymic browning and ascorbic acid oxidation, thereby providing more evidence that the relationship between Mm and food stability is one of considerable importance.

2.10.3 The Next Stage

From this starting point, things have moved rapidly, such that now Mm is accepted as one of the key determinants of food stability. The beginning of the modern approach to Mm emerged from pioneering studies by Franks [59] and Slade and Levine [60–65], who demonstrated that Ferry's concepts could be applied, in modified form, to the understanding of food stability. The important

advance was the postulation that aqueous sugar glasses and other materials in the glassy state in food systems could be conceived of as having properties similar to those of the amorphous polymers characterized by Ferry et al., and that relationships similar to those developed by Ferry could be applied to rate behavior in these "aqueous food glasses." In particular, Levine and Slade proposed the application of the Williams–Landel–Ferry (WLF) equation to food systems. The WLF equation takes the form

$$\log \frac{\eta}{\eta_g} = \frac{-C_1(T - T_g)}{C_2 + (T - T_g)} \tag{2.8}$$

where η is the viscosity at product temperature T (K), η_g is the viscosity at product temperature T_g (K) (usually the glass transition temperature) and C_1 (dimensionless), and C_2 (K) are constants. η can be replaced by 1/Mm, the molecular mobility, or any other diffusion-limited relaxation process. This equation describes the dependence of system viscosity, and other diffusion-enabled processes, on the amorphous state behavior of a polymer. In polymer systems, universal values have been established for C_1 and C_2. It is a topic of dispute as to whether these values can be usefully applied to systems of aqueous food glasses. While Levine and Slade coined the phrase "food polymer science approach" to describe these interrelationships, it is perhaps more helpful to focus on the underlying concept of molecular mobility, as we have chosen to do in this chapter.

2.10.4 Factors That Influence Reaction Rates in Solution

Before discussing the concepts of molecular mobilities as determinants of reaction rates in diffusion-limited systems, it is important to note that at ambient temperatures, chemical reactions in aqueous solution are often not diffusion limited. At conditions of constant temperature and pressure, three primary factors govern the rate of a chemical reaction, a diffusion factor, D, describing the probability of an encounter, a frequency of collision factor, A, defining the number of collisions per unit time following encounter, and a chemical activation energy factor, E_a, defining the energy barrier to be surmounted in a collision between properly oriented reactants. The latter two terms occur in the Arrhenius relationship describing the temperature dependence of the reaction rate constant. For a reaction to be diffusion limited, neither A nor E_a can be rate limiting; in other words, properly oriented reactants must collide with great frequency, and with a sufficiently low activation energy to ensure that collisions have a high probability of resulting in reaction. Therefore, diffusion-limited reactions typically have low activation energies (8–25 kJ/mol). Most "fast reactions" (small E_a, large A) are diffusion limited. Examples of diffusion-limited reactions are proton transfer reactions, acid–base reactions, many enzyme catalyzed reactions, protein folding, polymer chain growth, radical recombination reactions, and oxygenation/deoxygenation of hemoglobin and myoglobin. A rate constant of 10^{10} to 10^{11} M^{-1} $s^{-1}$is regarded as presumptive evidence of a diffusion-limited reaction. The diffusion-limited rate is the maximum rate possible for a reaction in solution (conventional reaction mechanisms, which are normal, are assumed).

The viscosity and temperature dependence of the diffusion constant is pertinent. The second-order diffusion-limited rate constant for uncharged spherical particles is given by the Smoluchowski's equation

$$k_{dif} = \frac{4\pi N_A}{1000}(D_1 + D_2)r \tag{2.9}$$

where N_A is Avogadro's number, D_1 and D_2 are diffusion coefficients for particles 1 and 2, and r is the distance of closest approach of particles 1 and 2, represented by the sum of their radii.

And, from the Stokes–Einstein equation:

$$D = kT/\pi\beta\eta r_s \tag{2.10}$$

where k is the Boltzmann constant, T is the absolute temperature, β is a numerical constant, η is the viscosity, and r_s is the hydrodynamic radius of the diffusing species.

Since viscosity increases rapidly as temperature is reduced in the WLF region, this dependence of D, and hence k_{dif} on viscosity is of particular interest.

It appears likely that the rates of some reactions in high moisture foods under ambient conditions are diffusion limited, while others are not. Those rates that are diffusion limited would be expected to conform with WLF kinetics as temperature is lowered, or as moisture content is decreased.

2.10.5 The Role of Molecular Mobility in Food Stability

The major concept regarding the relationship between molecular mobility and stability of foods is very simple. As a food is cooled, molecular mobilities decrease. This is a normal consequence of lowered temperature. The different molecular species of the food will, of course, each have their own characteristic mobilities. Two scenarios are possible. In the simpler scenario, as temperature is lowered, at some point the mobility of the larger molecules becomes so constrained that their diffusion becomes highly restricted and processes depending on their mobility will slow down markedly. At some lower temperature, intermediate size molecules also experience restricted motion, and the properties of the system, and its reactions, exhibit increased temperature dependence in the temperature zone where restricted motion occurs.

In the more complex scenario, as temperature is lowered, a new solid phase begins to separate. This is most relevant when the solid phase is ice (i.e., freezing). As ice separates out, concentration of solutes in the unfrozen aqueous phase increases. Molecular mobility is not solely a function of the temperature, it is also a function of the concentration, since at higher concentrations collisions and entanglements become more likely. Hence, in these systems, as temperature is lowered, molecular mobility is reduced both through the effect of temperature and also through the effect of increasing concentration. The combination of these factors leads to a reduction in mobility with decreasing temperature, which is much more pronounced than that that occurs when temperature lowering is the only driving force. As before, molecular size is a factor, with large molecules exhibiting severely restricted mobilities at higher temperatures than do smaller molecules. The patterns of behavior of frozen systems have been studied in great detail over the past 30+ years and are discussed further in terms of molecular mobilities later in this section.

Evidence suggests that Mm is causatively related to diffusion-limited properties of foods that contain, besides water, substantial amounts of amorphous, primarily hydrophilic molecules, ranging in size from monomers to polymers. The key constituents with respect to Mm are water and the dominant solute or solutes. Foods of this type include starch containing foods, boiled confections, protein-based foods, intermediate moisture foods and dried, frozen, or freeze dried foods.

When a food is in a condition where Mm is greatly reduced, diffusion-limited properties become quite stable, changing very slowly, or not at all with time. Note that while most processes of physical change are diffusion limited, not all processes of chemical change are so limited. Sometimes chemical reactivities are the dominant factor in food stability. Nevertheless, diffusion-limited processes often play an important role in food stability.

As we now discuss the Mm approach to food stability in more detail, one should consider this approach as a powerful complement to, rather than a replacement for, RVP as a tool to predict the stability of food systems containing water as a major component.

2.10.6 The State Diagram

2.10.6.1 Introduction

Since food systems rarely exist in equilibrium, traditional phase diagrams are of limited use for understanding the phase behaviors of foods. It is better to utilize an extended form of diagram, often termed a state diagram, which provides information on nonequilibrium, perhaps metastable "states," in addition to information on equilibrium phases. The term "state" refers to the nonequilibrium manifestation of what would, at equilibrium, be labeled a phase. Its use is encouraged, to distinguish clearly between equilibrium or nonequilibrium observations. State diagrams are in essence "supplemented" phase diagrams.

A simplified temperature-composition state diagram for a binary system is shown in Figure 2.23. Compare this to the phase diagram of Figure 2.10. In these diagrams, solid lines are assumed to be thermodynamically defined, while dashed lines indicate the locus of a property or parameter that is kinetically defined, or metastable. In general, these loci are plotted as the limit lines, defining the putative final reality of the property. It is assumed, in using these diagrams, that pressure is constant, and that the time dependence of metastable states is of little or no significance in the systems of

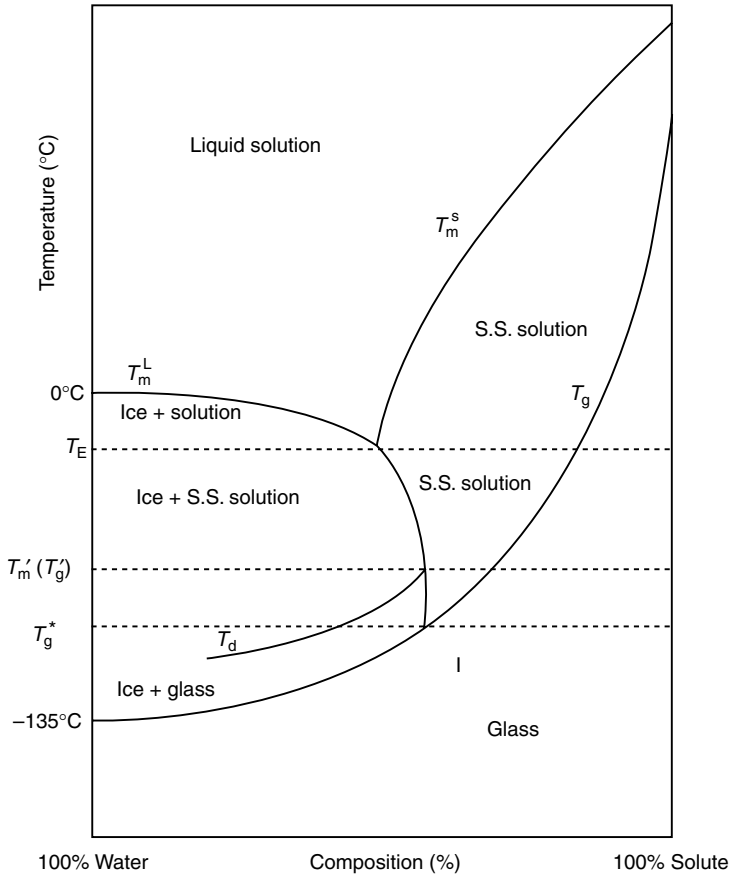

FIGURE 2.23 Annotated temperature composition state diagram for a binary aqueous system. The assumptions are of maximal freeze concentration, no solute crystallization, constant pressure, and no time dependence. T_m^L is the melting point curve, T_E is the eutectic point, T_m^s is the solubility curve, T_g is the glass transition curve, T_d is the glass devitrification curve, T'_m (T'_g) is the onset of melting, and T_g^* is the solute specific glass transition temperature of the maximally freeze concentrated solution.

interest. While all state diagrams for simple binary systems will have the same form, real, complex foods cannot be easily represented by a binary state diagram. However, providing the only material to crystallize is water, a binary state diagram can provide an adequate approximation of the state behavior of a complex food, and can display the glass transition curve with sufficient accuracy to be of value. To use such a diagram, one considers the totality of all nonaqueous components of the aqueous phase as though they were a single solute. This is acceptable providing no component of the nonaqueous solute mix separates out (crystallizes, precipitates, or forms a separate new liquid phase). Since in most frozen foods ice is the sole component to separate from the aqueous phase, a pseudobinary diagram can easily be obtained by plotting T_m^L as a function of concentration. It should be realized, however, that in a complex food, different regions of the food, or different component molecules, may exist in phases distinct from those of other regions or those containing other component molecules, and thus may require description by multiple binary state diagrams. It can be challenging to identify which is the dominant component or dominant region, controlling the critical properties, in such a system. For example, in some systems containing a mixture of polymers, phase separations occur, creating domains that may contain differing dominant polymer species. In such a complex system, identifying the most relevant glass transition, or mobility restriction, is difficult. The critical reactant may be in one, or several, of the many phases present.

2.10.6.2 Interpreting a State Diagram

It is now appropriate to discuss some of the constraints when interpreting state diagrams. The region of the diagram that represents the true phase diagram, indicated by solid lines, depicts the true equilibrium situation. Both the ice melting point line T_m^L and the saturation line T_m^s, and their intersection at point E, the eutectic point, describe true equilibrium situations. Line T_m^L is located by conducting cooling and warming cycles, with data collected from the warming experiments. Beyond E, the continuation of line T_m^L describes a new, and more complex, reality. First, it exists only if the solute has failed to crystallize (failure is common). In the absence of solute crystallization, the portion of the diagram to the higher concentration side of T_m^L represents a supersaturated (SS) solution. Thus, E to T_g^* represents a nonequilibrium state. The extension of the T_m^L line from E to T_g^* is usually taken to represent metastable equilibrium and represents the highest concentrations of solute in solution (supersaturated) that can be achieved by crystallization of ice at any temperature. It is possible to have, at any given temperature, less ice in the system, and hence a lower solute concentration, depending on the exact crystallization kinetics during cooling to that point. The more rapid the cooling, and the lower the temperature, the more likely incomplete ice crystallization becomes.

At some temperature a condition is reached where no further ice will separate on cooling. In the ideal diagram, this is represented by the intersection of line T_m^L with line T_g at T_g^*, with solute concentration C_g^*. Line T_g represents the temperature of the glass transition for a *homogeneous* amorphous matrix as a function of matrix composition. Note that in order to determine the entire line T_g from pure HOH to pure solute, cooling conditions must be applied in systems representing the entire range of concentration such that *neither* solute nor solvent can crystallize, as a result forming a series of homogeneous glasses of known concentration at appropriately low temperatures. Achieving this can present a challenge, particularly at the higher water concentrations, where very rapid cooling might be necessary to prevent any ice crystallization. Curve T_g for an amorphous aqueous system extends from $-135°C$ for pure water to whatever is the appropriate T_g for the pure solute.

In equilibrium systems, in which ice crystallization is taking place, the eutectic point E defines the concentration, C_E, that represents the critical concentration of the equilibrium system at which there is a transformation from ice crystallization alone to simultaneous ice and solute crystallization. Only at this unique solution phase concentration can ice and solute cocrystallize, and they do so at a constant ratio equivalent to the ratio of water to solute in the solution phase at concentration C_E. At higher initial water concentrations, solute concentration C_E is arrived at by ice crystallization and at higher initial solute concentrations by solute crystallizing.

Cryobiologists, who have an interest in the properties of dilute aqueous glasses, have studied aqueous glasses extensively [66–68]. Of particular interest to us is the observation that, on warming a homogeneous, dilute glass from a very low temperature, a glass transition is observed at T_g. On further warming, at some temperature T_d, devitrification occurs, with the formation of some ice, an exothermic process, and a concomitant increase in the solute concentration of the residual glassy phase to approach a maximum possible concentration $(C_g)_T$ defined by line T_g at temperature T_d. This result suggests that, at T_d, solute molecular mobility has increased sufficiently to allow for some spatial molecular rearrangement, and the formation of pure water domains in the form of ice. The resultant increased concentration within the residual solution phase domains results in decreased solute mobility, such that these domains remain effectively in the glassy state, with T_g appropriate to their increased solute concentration. The exact location of T_d will depend upon the experimental timescale. For longer timescales, T_d will be at a lower temperature. Note that, since ice crystallization is exothermic, devitrification will be a self-accelerating process, and therefore should be readily observed.

Consider now what happens as a system whose overall concentration is more dilute than the eutectic concentration, C_E, is cooled less rapidly than is required to form the homogeneous glass. For this exercise, we will assume that initial ice nucleation occurs readily (not always so in practice) such that there is no significant undercooling. As cooling progresses, the initial T_m^L for the starting concentration is reached and ice formation commences. On further cooling, more ice forms, and the resulting composition of the unfrozen phase tracks T_m^L. Eventually, point E is reached, and if equilibrium is maintained, solute and ice will cocrystallize at temperature T_E in constant proportion (defined by the proportion at C_E) until the whole system becomes solid. With further cooling, the temperature will again decline, but with no additional change in phase concentration (a vertical descent from E). However, nucleation and growth of solute crystals can be difficult. Supersaturation is common. Should supersaturation occur, continued cooling on reaching point E produces more ice, but no simultaneous solute crystallization, and therefore the composition of the unfrozen phase continues to increase beyond C_E, tracking along line T_m^L from E to T_g^*, at which point the unfrozen phase enters the glassy state. This concentration can be denoted as $(C_m^L)_T$. At T_g^*, $(C_m^L)_T$ is C_g^*. As was previously noted, on concentration increase and also on temperature decrease, solution viscosity increases. Hence, the mobility of solute molecules within the solution phase, particularly large solute molecules, decreases, and the segregation process required to form regions of pure water in the form of ice takes more time. The continuation of line T_m^L from E, as drawn, defines the *maximal* concentration $(C_m^L)_T$ possible for the unfrozen phase at each temperature. Should the rate of ice crystallization be restricted (e.g., by rapid freezing), the concentration of solute in the unfrozen phase will be less than $(C_m^L)_T$ at any given sub-T_E temperature. Eventually during continued cooling, this unfrozen phase will attain a temperature and concentration that coincides with some point on the line T_g, representing the glass transition of a more dilute glass than the maximally freeze concentrated T_g^* glass.

2.10.6.3 The Interplay of Equilibrium and Kinetics

Fixing the exact location of this intersection of the line T_m^L with line T_g has been a subject of considerable controversy. Levine and Slade identified, from DSC curves, a temperature they termed T_g', which they assumed to be the temperature of the glass transition of the maximally freeze-concentrated matrix. From their data, they attempted to calculate the concentration (W_g', the concentration of water, or C_g', the concentration of solute) at this point [69], but their initial calculations proved to be in error [70–72]. Later studies, determining T_g in higher concentration quenched systems, or using a revised method to estimate the ice content, were required to arrive at the estimates of W_g^* or C_g^*, respectively the water content of the glass and the concentration of the glass, in Table 2.5. Other workers, including Roos and Karel [73] and Simatos and Blond [74], have challenged Levine and Slade's characterization of the point they labeled T_g' as representing a glass transition. Evidence was

TABLE 2.5
C_g^* Estimates for Selected Carbohydrates

	C_g^* (wt%)		
Carbohydrate	**Levine and Slade**	**Other Workers**	T_m' **(K)**
Glycerol	54	80	208
Ribose	67	81	226
Glucose	70.9	80	230
Fructose	51	83	231
Galactose	56	83	232
Sucrose	64	81	241
Maltose	80	81	244
Maltotriose		81	250

Source: Slade, L. and H. Levine (1995) In *Food Preservation by Moisture Control* (G.V. Barbosa-Canovas and J. Welti-Chanos, Eds.), Technomic Press: Lancaster, PA, pp. 33–132.

presented suggesting that the true glass transition was seen at a lower temperature. It was suggested that the T_g' of the Levine and Slade terminology was really the initiation of melting, and it was proposed that the symbol T_m' be used to identify this point. This chapter follows this terminology, with T_m' representing the point initially labeled T_g' by Levine and Slade. Since Levine and Slade proposed that T_g' was the significant temperature to be used in the WLF equation when estimating reaction rates in frozen systems, this was not a trivial dispute. It has since been resolved by realizing that, indeed, this important temperature probably represents the point at which, on warming, the melting process for ice emerges from the kinetic constraints of reduced solute mobility, and follows line T_m^L defining the metastable system. It may therefore be considered as the final point of the limit T_d curve, representing the changeover from mobility constraints on ice crystallization to mobility constraints on ice melting. Line T_d represents the highest temperature at which an effective kinetic constraint on ice crystallization in a nucleated system at sub-T_m temperatures is found to exist. Combining concepts, the symbol T_m' could be considered to represent the "mobility temperature" at which the constraints due to reduced solute mobility are overcome during warming. It represents the temperature where diffusion of solute in the maximally freeze-concentrated system can occur at a reasonable rate. As will be discussed later, this temperature is an important parameter when estimating stability and reaction rates in frozen systems maintained at subfreezing temperatures above T_m'. Since T_m' clearly represents the point at which solute mobility first becomes sufficient to observe measurable change, it is an appropriate reference temperature to use in a WLF equation estimating mobility increase (and its consequences). In the WLF equation, the reference temperature is assumed to represent a repeatable mobility threshold for a variety of systems. Clearly, using T_m' to represent the temperature at which the system has its reference mobility uses the initiation of solute diffusion sufficient to enable ice melting as the criterion. This provides support for the proposal by Levine and Slade that T_m' was the appropriate temperature to use in the WLF equation when attempting to predict frozen product stability. Note that the concentration of the limit unfrozen system at T_m' will be very close to C_g^*, since until solute mobility is sufficient to allow for ice melting, and dilution of the unfrozen matrix, there should be no change in the concentration of the unfrozen matrix. It should be remembered that this discussion refers to the ideal of the limit metastable systems. In reality, temperatures and concentrations will be determined in a range around T_m' and C_g^*. The term T_g^* is used in this chapter to represent the temperature of the glass transition of the maximally freeze-concentrated glass, in preference to T_g', to avoid unnecessary confusion, given the multiple definitions that have been assigned to T_g' in the literature.

2.10.6.4 Extending the Concept to Complex Food Systems

An approach with some merit is to identify the dominant food solute within a system, then deduce the properties of the complex food from the binary state diagram for this solute. An important useful example is the use of a sucrose–water state diagram to predict the properties and behavior of cookies during baking and storage [75]. If in other situations more than one primary solute is present, a state diagram for each solute should be considered. It should be borne in mind that the state diagram is constructed from observations of the temperatures of phase and/or glass and related transitions (such as devitrification) as a function of the moisture content of the system. In single solute (or fixed solute ratio) systems that can form ice, T'_m and T^*_g are the same for all starting compositions (though T_d and T_g are composition dependent), and the changing liquidus temperature (T^L_m) can be tracked by following, as discussed, the change in amount of ice (and hence C^L_m, the concentration of the liquid phase in contact with ice) at each temperature. However, in binary systems with water contents lower than that of the maximally freeze-concentrated matrix (and hence unable to form ice), separate determinations of all the transition temperatures are required for each moisture content. At low moisture content, identification of T_g is usually feasible because solute seldom crystallizes. However, determination of the saturation line, T^s_m, is often difficult because of the reluctance of solutes to crystallize at or to their saturation concentration. In complex systems, where no dominant solute (DS) can be identified, such as in dry or semidry complex foods, determining the T^s_m curves is not yet possible. It is, however, as discussed above, a relatively simple task to determine T^L_m and hence it is possible to determine a state diagram for a complex frozen food. Figure 2.24 indicates how the state diagram changes for different solutes.

One final comment should be made. While, at temperatures above T_E, T^L_m, and T^s_m are indeed lines, T_g and T_d are descriptors of kinetic barriers, and better considered as bands. The exact event temperature is dependent upon the "characteristic frequency" or inherent response time of measurement, as one might expect for a kinetic process [76,77].

Examining the state diagram for solute concentrations greater than the maximal freeze concentration, the T_g curve is seen to rise with increasing steepness toward T^s_g, the T_g of the solute. Described differently, even a small amount of water present in the solute greatly influences T_g. This phenomenon, known as plasticization, is a property of all glasses to which are added amounts of significantly smaller molecules than the molecules whose mobility defines the glass transformation. A widely accepted explanation for plasticization involves consideration of free volume, the space between the major molecules of the glass. Should this free volume increase, it is clear that molecular movement would become easier, corresponding to a lowered glass transition temperature. Small molecules can enter the interstices between larger molecules, thus increasing the free volume, and consequently lowering T_g. Water, as a very small molecule, is an effective plasticizer. Other molecules, such as ethanol and glycerol, can also provide plasticization, assuming that they can enter the phase occupied by the molecules that constitute the amorphous phase. Should the small molecule not be miscible with the larger molecule on the molecular level, then plasticization as described here cannot occur, though a lubrication effect between separate "microphase domains" might still occur. Such lubrication would influence mechanical properties, but the thermal properties of the system would still reflect the T_g of the domains, and the kinetics would be expected to conform to the T_g of the domains also.

2.10.6.5 Identifying the Assumptions

The applicability of this molecular mobility approach to food stability is dependent upon several key concepts and assumptions. The first, and most important premise, is that *many (if not most) foods contain amorphous components, and exist in either a state of metastable equilibrium or in a nonequilibrium, kinetically labile state.* This is true of many complex foods because most of them contain amorphous solid, and supersaturated liquid regions. Biopolymers are typically at least partly

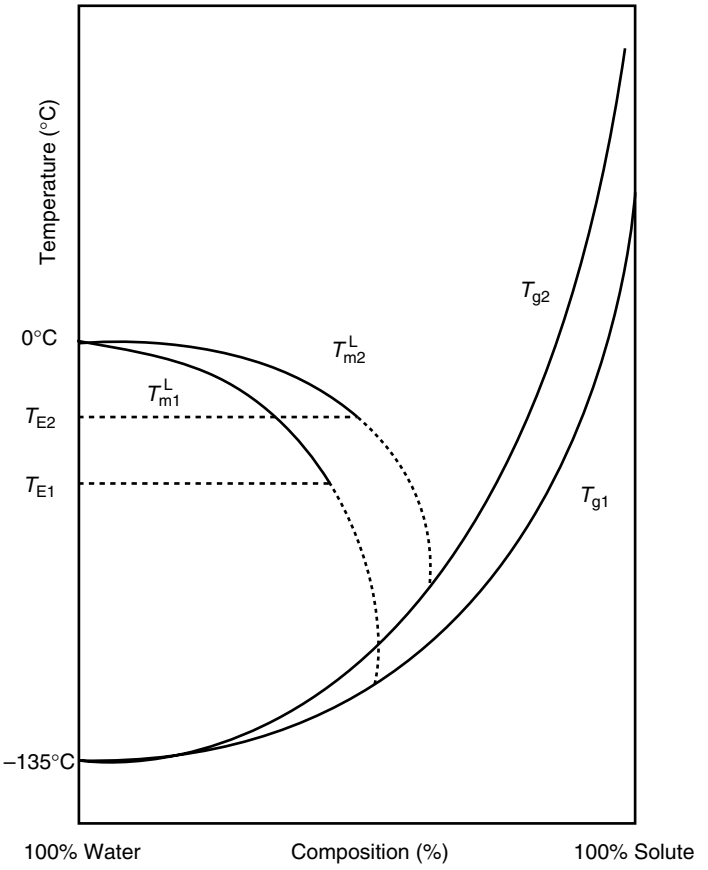

FIGURE 2.24 State diagrams for binary systems showing the influence of solute type on the position of T_m^L and on T_g. The extreme left of T_g is fixed at the vitrification temperature of pure water (−135°C). The assumptions stated in Figure 2.23 apply here.

amorphous. Many small molecules are difficult to crystallize from solution, and thus exist in an amorphous state when in excess of saturation. This amorphous state may be kinetically constrained, and therefore metastable, or may be slowly changing with time, and therefore in nonequilibrium. Essentially, the lower (T_g) boundary line in a state diagram defines the limiting conditions for the metastable amorphous phase, and the region of $C > C_E$ above T_g and below T_m^s represents a nonequilibrium amorphous state with the upper boundary of this condition being defined by the saturation solubility line (T_m^s) above which exists the simple solution. Note that the part of T_m^L to the left of point E ($C < C_E$) (Figure 2.23) similarly defines the upper limit of the nonequilibrium amorphous state for compositions where ice is not present owing to rapid cooling, or other constraints. Since it is more difficult to prevent ice crystallization than it is to prevent solute crystallization in foods, the amorphous state for compositions to the left of point E is not readily attained, except for (1) the region of initial undercooling prior to the initial nucleation of ice and (2) the region with C^L less than, but close to C_m^L as defined by the liquidus representing an unfrozen phase that can be termed a nonmaximally freeze-concentrated matrix. It is a major goal of food scientists and technologists to maximize the number of desirable food attributes that depend on metastable equilibrium states, and to find conditions providing acceptable stability for those desirable attributes that depend on the maintenance of nonequilibrium states.

The next key point is to reiterate that *the rates of most physical processes, and also of many chemical processes, are governed by molecular mobility*, in that they require some form of molecular

motion to be accomplished. Since, as we have discussed, most foods exist in metastable or nonequilibrium states, kinetic approaches are often most relevant to the understanding, prediction, and control of their properties. Molecular mobility provides an appropriate kinetic approach, as it is causally related to the rates of diffusion-limited processes in foods. The use of the WLF equation, to provide estimates of Mm at temperatures above that of the glass transition, T_g, but below T_m^L has been established as a standard procedure over the last decades. State diagrams usefully define regions of temperature and composition that permit metastable or nonequilibrium conditions to exist for useful periods of time. In frozen systems, a particular controversy has addressed the issue of which T_g to employ in the WLF equation. As has been noted, while Levine and Slade recommended the use of T'_m (T'_g) in their terminology, it is clear that this does not take into account the dilution of the unfrozen phase due to melting at the higher temperature. Nor does it acknowledge that the true glass transition temperature of the maximally freeze-concentrated matrix is T_g^*. As has been suggested by several workers [74,78,79], it would appear to be more correct to use T_g, the temperature of the glass transition for this more dilute phase. However, given that the parameters of the WLF equation do not have universal values, it emerges that the use of either convention is equally effective in real systems [79]. Given that establishing the true value of T_g is a major challenge, it is indeed fortunate that, as indicated earlier, use of T'_m in the WLF equation provides sufficient accuracy.

2.10.7 Limitations of the Concept

While the Mm approach is useful for predicting many kinds of physical change, its utility is not universal. Examples of where it is unsatisfactory include chemical reactions whose rates are little influenced by diffusion, effects achieved through the action of specific chemicals, and situations where the estimated Mm reflects the properties of a polymeric component, while the process of concern involves smaller molecules whose mobility is little hindered by the loss in mobility of the primary matrix. Also, in the growth of vegetative cells of microorganisms, the mobility of water, and consequently $(p/p^o)_T$ serves as a better predictor.

Returning to the discussion of reaction kinetics, in the last 20 years, there has been an active discussion as to whether the WLF equation or the Arrhenius equation provides the better description of the temperature dependence of reaction kinetics in aqueous food systems, particularly at temperatures between T_g and T_m^L or T_m^s. Consider systems that can form ice. In this region, in taking the molecular mobility approach, there are two factors that might be expected to influence mobility, temperature, and concentration. As temperature is lowered, concentration increases. At first the influence on mobility is primarily that of temperature, but as the temperature continues to drop, the increasing concentration becomes a factor of increasing importance as more ice forms. Figure 2.25 illustrates the effect of temperature and the effect of concentration separately upon viscosity and mobility. The combined effect is shown in Figures 2.26 and 2.27. Both the Arrhenius equation and the WLF equation properly describe the effects of temperature on kinetics only if concentration is constant. The effect of concentration on kinetics enters as another term in the analysis. For a first order reaction, concentration does not influence the *fractional rate* of reaction (i.e., $t_{1/2}$ is independent of concentration), but for all higher orders of reaction, the relative rate of reaction is concentration dependent. For many reactions in frozen systems, a pseudo first order description is adequate, but this does not guarantee that, especially in the freeze-concentrated zone, the effect of concentration can be ignored when estimating extent of reaction. As previously noted, empirical evidence shows that an equation of the WLF form can provide adequate estimates for rate and extent of reaction as a function of temperature and time, using either T'_m, T_g^*, or the T_g of the homogeneous glass of composition C_T^L (where T is the storage temperature of interest) as the reference temperature. Given the limited range of temperatures in the region between T_g and T_m^L in frozen systems, it is not surprising that the Arrhenius equation also produces a satisfactory fit to the raw data.

Note that another factor that is little discussed is the "equimolal" nature of the unfrozen phase in frozen systems. The presence of ice defines the osmolality of the unfrozen phase, assuming that

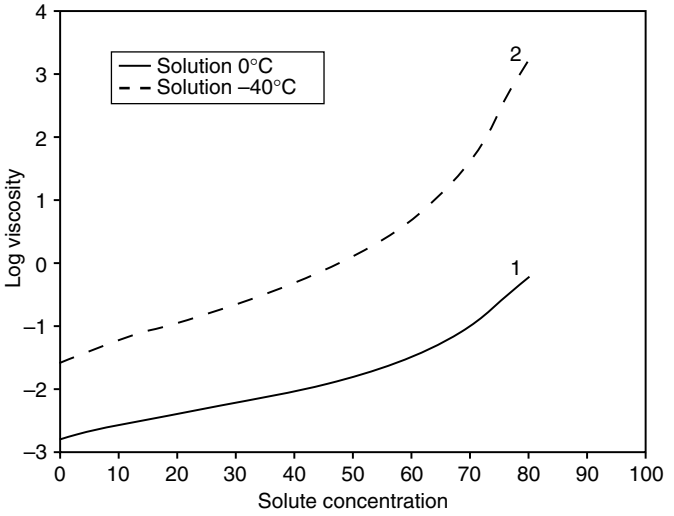

FIGURE 2.25 Comparison of the effect of concentration on the viscosity of aqueous solutions at two different temperatures: (1) 0°C and (2) −40°C.

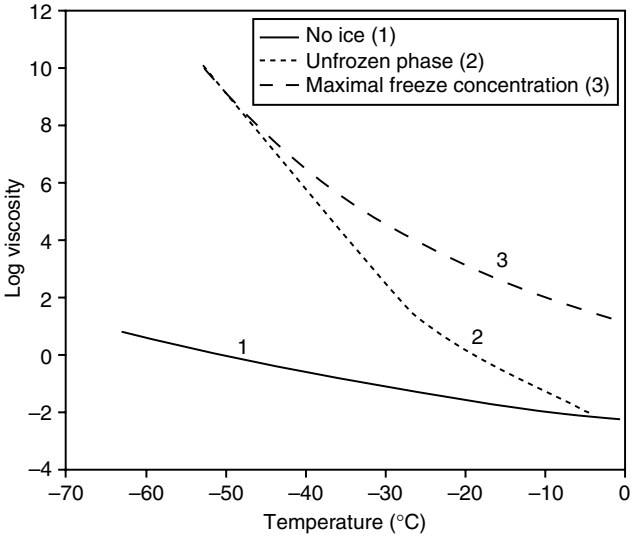

FIGURE 2.26 Predicted viscosities in aqueous systems as a function of temperature: (1) no ice formation on cooling; (2) ice separation such that solution phase concentration tracks line T_m^L; (3) system concentration is C_g^*.

T_m^L defines the concentration. Should the composition (and hence mole ratios) of solutes change as a consequence of reactions, in contrast to an unfrozen system, the amount of ice, and hence the individual concentrations, will adjust to maintain the defined osmolality of the unfrozen phase. Hence, the evolution of reactant and product concentrations could depend upon the stoichiometry of the reaction in a way different from that of an unfrozen system.

In a system of concentration in excess of T_g^*, where ice crystallization is not possible, above T_m^s, in the fluid system, Arrhenius kinetics hold. It is not uncommon for an Arrhenius plot incorporating temperatures that span T_m^s to exhibit a change in slope around T_m^s. Between T_m^s and T_g the system can be described as rubbery. Particularly note that there is a rapid decrease in mobility as the temperature is lowered, and this is reflected by a rapid change in reaction rates. In this region, while it is difficult

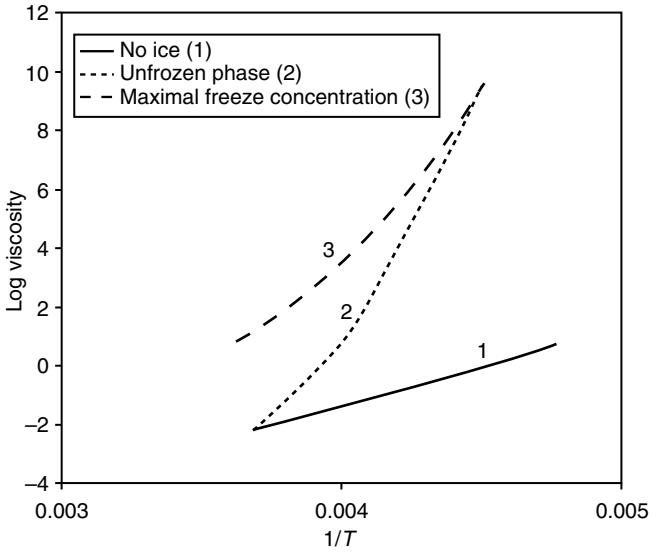

FIGURE 2.27 Reciprocal temperature plots of the data of Figure 2.26.

to come up with a uniform approach, it has been noted that the rates of many physical events conform more closely to an equation of the form of the WLF equation than to the Arrhenius equation. For chemical reactions, the dependence on molecular mobility is very dependent on reaction type, and neither WLF nor Arrhenius kinetics describes all reactions in this zone. Note that this discussion does not apply to frozen foods (concentrations less than C_g^*), as it fails to account for the influence of increasing concentration of the unfrozen phase, as ice content increases, on the properties of the unfrozen matrix, illustrated in Figures 2.26 and 2.27.

2.10.8 Practical Applications

2.10.8.1 Developing the State Diagram

Having discussed in some detail the applicability of the state diagram, and the WLF equation, in understanding product stability, it is appropriate to discuss in more detail the challenges involved in determining the state diagram and also in the application of the WLF equation. The state diagram is constructed by, instrumentally, identifying temperatures of "change" for systems at a wide range of concentrations. Techniques such as DSC, DMTA have proved useful, but require special instrumentation, and are demanding to interpret. In each case, an instrument response is plotted as a function of temperature, and the temperatures at which breaks in the trends of the response occur are plotted. While, as Levine and Slade initially demonstrated, it is possible to obtain an estimate of T'_m for many systems by determining the DSC melting curve for frozen solutions of overall concentration of 20% solute, 80% water, the determination of the concentration of the maximally freeze-concentrated phase from such data is challenging, as is the precise determination of T'_m. Considering first the challenge of determining T'_m, it is better to study initial solutions of a range of concentrations, as in this way the influence of instrumental artifacts and also slow crystallization can be minimized. Temperature cycling also provides a useful tool, as cycling above and below T_g^* and also around T'_m assists in approaching the formation of maximal ice during the cooling cycles. Obtaining a consistent value for T'_m from a range of initial solution concentrations allows a level of confidence to be attached to the value. The estimation of C_g^* is subject to greater difficulty. The approach initially used by Levine and Slade was to estimate the amount of ice present in the system after formation of the maximally freeze-concentrated glass. Unfortunately, their initial method for estimating the ice

content had a serious flaw. Using solutions of initial concentration 20% in solute, the ice content was determined from the area of the peak corresponding to ice melting in a warming thermogram. To do this, Levine and Slade assumed the melting enthalpy of ice to be that of ice at 0°C. Unfortunately, the melting enthalpy of ice is temperature dependent, decreasing as temperature is lowered. Hence this method leads to an underestimation of the quantity of ice, and therefore a value for C_g^* that is too low. Other workers have suggested means of better estimating the ice content, allowing for the temperature dependent enthalpy of fusion, and have also suggested that methods that employ several initial solution concentrations are more robust, since, as in the estimation of T'_m, agreement among results obtained from samples of a range of initial concentration generate greater confidence in the reliability of the estimate [72,80–82]. As with T'_m, application of a temperature cycling protocol prior to the final warming scan used for the estimation of the ice content leads to a closer approach of the system to that containing the maximally freeze-concentrated glass, and therefore a better estimate of C_g^*. Determining T_g for a glass of the composition of the initial solution is a much greater challenge than determining T'_m. As discussed previously even with very rapid cooling it is difficult, except at initial concentrations close to C_g^*, to prevent all ice crystallization. Hence it is often necessary to estimate, rather than measure, T_g for a particular concentration of solute. Various equations exist for this purpose. The oldest and simplest of these is that of Gordon and Taylor, which for a binary system is

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \tag{2.11}$$

where w_1 is the weight fraction of species 1, w_2 is the weight fraction of species 2, T_{g1} is the glass transition temperature for pure species 1, T_{g2} is the glass transition temperature for pure species 2, and k is a constant.

The initial development of the molecular mobility approach to food stability, by Franks, Levine, and Slade, focused primarily on understanding the behavior of predominantly carbohydrate systems. Many important concepts have been derived by looking at the behavior of carbohydrates, which are major food components. Empirically, for frozen systems, T'_m was found to depend significantly on the molecular weight of the solute. As molecular weight increases, T'_m increases. There is a smaller effect of molecular characteristics for sugars of the same molecular weight. A plot of T'_m vs. molecular weight for sugars, glycosides, and polyols (Figure 2.28) shows T'_m to increase

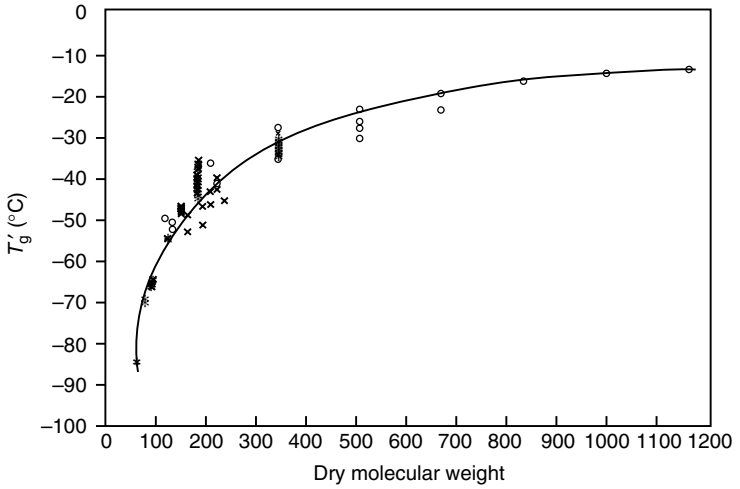

FIGURE 2.28 Typical results from Levine and Slade for T'_g (T'_m) as influenced by solute molecular weight: of solutions of sugar (o), glycoside (×), polyol (∗).

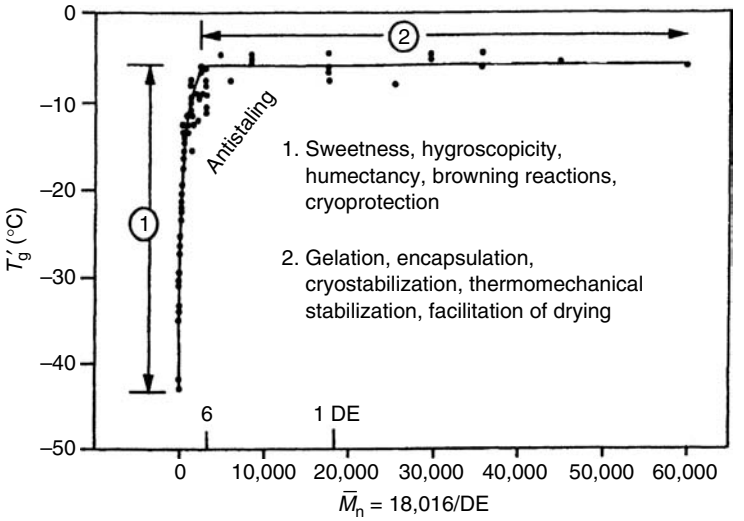

FIGURE 2.29 Typical results from Levine and Slade on the influence of dextrose equivalent (DE) and number average molecular weight of commercial starch hydrolysis products on T'_g.

proportionately with increase in solute molecular weight. This is to be expected, as translational mobility of molecules decreases with increasing size, so that a large molecule requires a higher temperature for movement than does a small one. At molecular weights above 3000 T'_m appears to become independent of molecular weight (Figure 2.29). Some data collected by Levine and Slade for sugars are reported in Table 2.6. Data are also reported for the dry sugars. Extreme care is required in collecting these data, since even slight traces of moisture can significantly lower T_g through plasticization. Assuming acceptable dryness, the T_g of the dry sugars is also found to depend upon molecular weight, as well as the nature of the individual sugar.

The concentration of the maximally freeze-concentrated matrix, C^*_g depends to some extent upon molecular size, but exact measurement can be, as has been indicated, a challenging problem. Assuming molecular mobility to be an important factor, differences in T'_m, and more particularly C^*_g, for molecules of the same molecular weight could result in very different effects on product stability. This presumably explains in part the differing efficacies of glucose vs. fructose and lactose vs. trehalose as stabilizing sugars.

2.10.8.2 The Freezing Process, Frozen Foods

Freezing preservation is recognized as one of the best methods for the long-term preservation of foods. A primary factor in providing the long-term stability is the low temperature, since, as has been emphasized earlier, reaction rates tend to decrease as temperatures are reduced. The formation of ice in frozen foods is somewhat of a two-edged sword. There are two important adverse consequences of ice formation in cellular foods and food gels. First the nonaqueous components become concentrated in the unfrozen phase and second there is a volume increase of around 9% associated with the transformation from liquid water to ice.

During freezing, water transforms to ice of high purity. Hence, solutes in the aqueous phase coexist with a decreasing amount of solvent water. This process is akin to regular dehydration, except that the temperature is lower, and that the separated water remains locally, but in the form of ice, rather than being physically removed from the local environment. The composition of the unfrozen phase at any temperature approaches that defined by the appropriate state diagrams.

As the freeze-concentration process progresses, the unfrozen phase changes significantly in its properties such as pH, titrable acidity, ionic strength, and so forth. Should solutes crystallize,

TABLE 2.6
Glass Transition Values and Associated Properties of Pure Carbohydrates

Carbohydrate	MW	Properties Dry			Properties Aqueous
		T_m (K)	T_g (K)	T_m/T_g	T'_m (K)
Glycerol	92.1	291	180	1.62	208
Xylose	150.1	426	282–287	1.49	225
Ribose	150.1	360	260–263	137	226
Glucose	180.2	431	304–312	1.39	230
Fructose	180.2	397	280–290	1.39	231
Galactose	180.2	443	303–305	1.45	232
Sorbitol	182.2	384	269–271	1.45	229
Sucrose	342.3	465	325–343	1.40	241
Maltose	342.3	402	316–368	1.19	243
Trehalose	342.3	476	350–352	1.35	243
Lactose	342.3	487	374	1.37	245
Maltotriose	504.5	407	349	1.17	250
Maltopentose	828.9		398–438		257
Maltohexose	990.9		407–448		259
Maltoheptose	1153.0		412		260

Source: Levine, H. and L. Slade (1988) In *Food Structure—Its Creation and Evaluation* (J.M.V. Blanshard and J.R. Mitchell, Eds.), Butterworths: London, pp. 149–180 and Slade, L. and H. Levine (1995) *Adv. Food Nutr. Res.* **38**: 103–269.

solute ratios will change, and pH can shift markedly. Also, dissolved gases may be expelled. At the higher, freeze-induced concentrations, macromolecules, forced into proximity, may aggregate. As has previously been mentioned, even though the effect of temperature per se is to reduce reaction rates, overall reaction rates, especially at high subfreezing temperatures, may increase or decline less than expected as a result of the higher concentrations of reactant resulting from freeze concentration. This can occur even though the total amount of reactant in a particular sample remains unchanged from the initial amount. Under such complex conditions, it is not surprising that reaction rates at subfreezing temperatures do not always conform to either Arrhenius or WLF kinetics. At low subfreezing temperatures, the higher concentrations resulting from freeze concentration, and the nearness to T'_m usually lead to reduced reaction rates, and enhanced storage life.

It is instructive to follow the freezing process in frozen foods in more detail with the aid of appropriate state diagrams. Consider first the slow freezing of a complex food. Slow freezing allows for close conformance to solid–liquid equilibrium and a close approach to maximal freeze concentration. Starting at A in Figure 2.30, removal of sensible heat moves the product to B, the initial equilibrium freezing point of the sample. Because nucleation is a difficult process, further removal of heat results in undercooling, rather than freezing, until point C is reached, where nucleation begins. Crystal growth immediately follows nucleation, releasing the latent heat of crystallization and causing the temperature to rise to D. Further removal of heat causes additional ice formation and the concentration of the unfrozen phase tracks along line T_m^L from D to E, at T_E. In a complex food, T_E represents T_{Emax} for the solute with the highest eutectic point (i.e., the lowest solubility). Solutes in complex frozen foods rarely crystallize at or below their eutectic points. An occasional exception is the crystallization of lactose in some frozen desserts, which leads to the textural defect known as "sandiness."

Assuming eutectics do not form, further ice formation leads to the metastable supersaturation of many solutes, with the composition following the line from E to the point F, the recommended

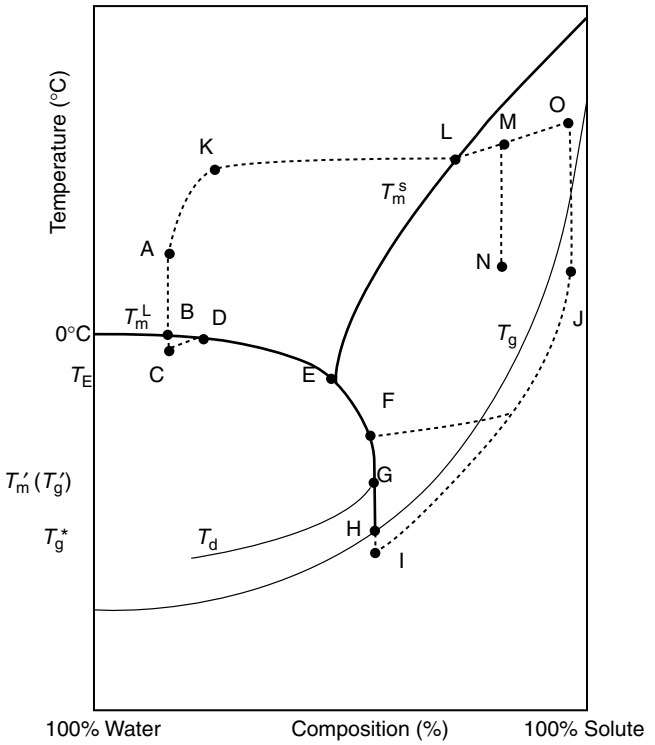

FIGURE 2.30 State diagram of a binary system showing possible paths for freezing (unstable sequence ABCDEF; stable sequence ABCDEFGHI), drying (unstable sequence AKLMN; stable sequence AKLMOJ), and freeze drying (unstable sequence ABCDEFJ; stable sequence ABCDEFGHIJ). The temperature scale is schematic to facilitate data entry.

storage temperature. For most foods, F is above T_m', indicating that molecular mobilities will still be quite large, and that the food's diffusion-limited physical and chemical properties will still be highly temperature dependent. Empirically, it has been found that rates of change are proportional to the temperature difference $(T - T_m')$ within about 20°C of T_m'.

If cooling continues beyond F, additional ice formation and freeze concentration occur, until the concentration reaches that at T_m' (point G). Further cooling does not lead to any additional ice formation, and at temperature T_g^* (point H) the supersaturated unfrozen phase converts to the glassy state, in which are embedded to ice crystals formed during the cooling process. T_g^* is a quasi-invariant T_g, being that of the maximally freeze-concentrated unfrozen matrix. T_g^* depends on solute ratios in the sample, but not initial solute concentrations. Maximal freeze concentration seldom occurs on the initial cooling cycle. Hence not only does the observed T_m' depend on solute ratios, but also to some extent on initial water content of the sample, as attainment of maximal freeze concentration is influenced by a range of kinetic factors. Appropriate temperature cycling around T_m' usually leads to a closer approach to C_g^* and hence the lowest measured T_m'.

It is important to note that the failure of ice to crystallize is not a consequence of reduced mobility of the water. These water molecules are observed to be still mobile and they freely exchange. The limiting process is the failure of solute molecules to translate or rotate in the applicable timescale, thus preventing the addition of further water molecules to the existing ice. In this situation (rapid freezing) water molecules are still exchanging on and off the ice.

As T_m' represents the temperature below which removal of ice from the matrix (on cooling) or dissolution of ice into the matrix (on warming) first becomes feasible in the timescale of our measurements, it defines a temperature at which Mm is greatly reduced during cooling and

diffusion-limited properties are found to exhibit excellent stability. Some T'_m values for various materials are shown in Tables 2.6 through 2.9. The question arises as to what happens when higher cooling rates are employed. Figure 2.31 illustrates the effect of increased cooling rate. Initially, after some undercooling, the unfrozen phase would be expected to track T_m^L. As the temperature declines, and the concentration of the unfrozen phase increases, Mm of the solutes decreases. The time required to produce clusters of pure water, at the same time rejecting solute from the volume to be occupied by the water, may exceed the time available. Hence less ice forms than is required by the T_m^L line, and the concentration of the unfrozen liquid phase, C_T^L at temperature T is less than would be predicted by the T_m^L line. Since the latent heat that would be associated with formation of this "missing" ice is not released, with continued removal of heat, the rate of reduction of temperature

TABLE 2.7
T'_m and DE for Selected Commercial Starch Hydrolysis Products (SHP)

SHP	Manufacturer	Source	DE	T'_m (K)
Staley 300	Staley[a]	Corn	35	249
Maltrin M250	GPC[b]	Dent corn	25	255
Maltrin M150	GPC	Dent corn	15	259
Paselli SA-10	Avebe[c]	Potato	10	263
Star Dri 5	Staley	Dent corn	5	265
Crystal gum	National[d]	Tapioca	5	267
Stadex 9	Staley	Dent corn	3.4	268
AB 7436	Anheuser-Busch	Waxy maize	0.5	269

[a] A.E. Staley manufacturing.
[b] Grain Processing Corporation.
[c] Avebe America.
[d] National Starch Corporation.

Source: Levine, H. and L. Slade (1988) In *Food Structure—Its Creation and Evaluation* (J.M.V. Blanshard and J.R. Mitchell, Eds.), Butterworths: London, pp. 149–180.

TABLE 2.8
T'_m for Selected Proteins

Protein	T'_m (K)
Bovine serum albumin	260
Lysozyme	256
α-Lactalbumin	262
α-Casein	260
Sodium caseinate	263
Gelatin 300 bloom	263
Gelatin 250 bloom	262
Gelatin 175 bloom	261
Gelatin 50 bloom	260

Source: Levine, H. and L. Slade (1990) In *Thermal Analysis of Foods* (V.R. Harwalkar and C.-Y. Ma, Eds.), Elsevier Applied Science: London, pp. 221–305.

TABLE 2.9
Estimates of T'_m for Selected Foods

	T'_m (K)			T'_m (K)	
Food	**Levine and Slade[a]**	**Other Workers**	**Food**	**Levine and Slade[a]**	**Other Workers**
Dairy			*Vegetable*		
Cottage cheese	252		Broccoli	246	252[d]
Cheddar cheese	249		Cauliflower	248	253[d]
Cream	250		Potato	257–262	
Ice cream	232–246		Spinach	256	239[d]
Skim milk	246	241[b]	Sweet corn	259–265	
Whole milk	251		Tomato	232	
Fresh fruit			*Meat and fish*		
Apple	231	245[d]	Beef muscle		261[c], 260[d]
Banana	238		Pork muscle		257[d]
Blueberry	232		Chicken		251[d]
Peach	237	244[d]	Turkey		253[d]
Strawberry	232–239		Cod muscle		262[c], 256[d]
Fruit juice			Catfish		256[d]
Apple	233	243[d]	Pollock		261[d]
Lemon	230		Salmon		256[d]
Orange	236	243[d]	Shrimp		241[d]
Pear	233				
Pineapple	232	241[d]			

[a] Levine, H. and L. Slade (1990) In *Thermal Analysis of Foods* (V.R. Harwalkar, and C.-Y. Ma, Eds.), Elsevier Applied Science: London, pp. 221–305 and Levine, H. and L. Slade (1989) *Comments Agric. Food Chem.* **1**: 315–396.
[b] Jouppila, K. and Y.H. Roos (1994) *J. Dairy Sci*. **77**: 2907–2915.
[c] Brake, N. and O. Fennema, unpublished.
[d] Hsu, J. and D. Reid, unpublished.

increases, further reducing Mm, and resulting in a greater and greater deviation of C_T^L from that defined by the T_m^L line. The deviations of C_T^L from the predictions of the T_m^L line increase with rate of cooling and with reduction in temperature.

2.10.8.3 Drying Processes

The state diagram of Figure 2.30 can also be used to illustrate the progress of many other processes involving changes in the state or amount of water in a system. Using it, the differences between air drying and vacuum freeze drying can be better appreciated.

First consider air dehydration at constant temperature. Starting from A, air drying will elevate temperature and remove moisture, until soon the product attains properties described by point K (the wet bulb temperature of the air). Further moisture removal causes the product to arrive at, and pass through, point L, on the solubility curve. At this point, saturation for the DS is reached. Crystallization does not immediately occur, and the product becomes supersaturated in DS, and any other solute with saturation temperature above DS. Each of these supersaturated solutions can be considered as an amorphous liquid phase. With continued removal of water the system can pass through point M, corresponding to the dry bulb temperature of the air, and on further to point O. Cooling the system at point M leads to N, which is above the T_g curve, and cooling from point O leads to J, which is below the T_g curve. This suggests that drying should be continued beyond the dry bulb air temperature (M) since should drying be terminated at M the product, at N, being above

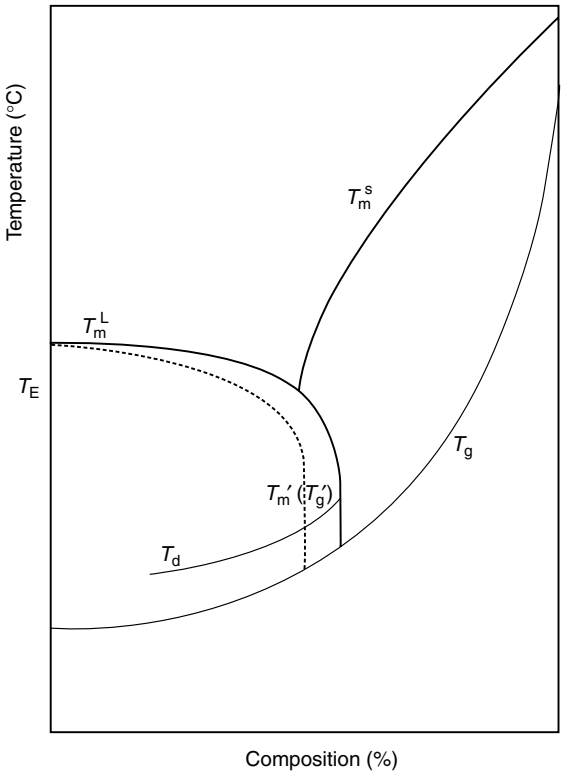

FIGURE 2.31 The effect of more rapid cooling on the composition and state of the unfrozen phase.

T_g will have relatively high Mm and consequent poor stability of diffusion-limited properties, which are strongly temperature dependent (WLF kinetics). Terminating drying at O, leads, at J to a product below T_g with a much reduced Mm, stable diffusion-limited properties, with only weak temperature dependence.

Product paths for vacuum freeze drying are also illustrated in the figure. The first stage of freeze drying coincides fairly closely to the path for slow freezing, ABCDEF. Should the product temperature not be allowed to go below temperature F during sublimation (primary freeze drying), path FJ would be typical. The initial stages of FJ involve ice sublimation. At some point with concentration around C_g^* ice sublimation will be complete and a desorption phase is entered. Since the sample is above the T_g curve, collapse is possible, particularly in products that were initially fluid, though the possibility exists to some extent for food tissues. Collapse is possible because no ice is present to provide structural support, and the product T is above T_g, and possesses sufficient Mm to preclude rigidity. Collapse results in a product of less than optimum quality. There is decreased product porosity, resulting both in slower drying and poorer rehydration characteristics. To prevent collapse the path ABCDEFGHIJ must be followed, where the portion HI represents cooling below T_g^*.

Provided maximal ice crystallization (maximal freeze concentration) has occurred, the critical temperature for structural collapse, T_c, the highest temperature at which collapse can be avoided during the primary stage of freeze drying, will lie somewhere between T_m' and T_g^*. The exact temperature will depend upon the rate of the drying process and hence the time period during which collapse would be feasible. The slower the process, the lower will be T_c. Should ice crystallization not be maximal, the highest temperature at which collapse can be avoided will approach T_d.

If the composition of the product to be freeze dried can be adjusted, it is desirable to raise T_m' as much as possible. This can be achieved by adding high molecular weight polymers, which enable

higher freeze-drying temperatures to be used. Increasing C_g^* (which increases the amount of ice formed) also enhances structural rigidity and minimizes the potential extent of collapse.

2.11 MOISTURE SORPTION ISOTHERMS

The molecular mobility approach is not the only approach to understanding food stability as a function of water content. Given the ability to determine both water content and RVP, it is instructive to consider the information that can be derived by examining the apparent dependence of water content upon RVP. This approach predates the molecular mobility approach by many years.

2.11.1 Definitions and Zones

A plot of water content (expressed as a mass of water per unit mass of dry material) of a food vs. $(p/p^o)_T$ is known as a moisture sorption isotherm (MSI). Information derived from MSIs are useful (1) for studying and controlling concentration and dehydration processes, because the ease or difficulty of removing water is related to RVP, (2) for formulating food mixtures so as to avoid moisture transfer among the ingredients, (3) to determine the moisture barrier properties needed in a packaging material required to protect any particular system, (4) to determine what moisture content will curtail growth of microorganisms of interest within a system, and (5) to predict the chemical and physical stability of foods as a function of changes in their water content.

Shown in Figure 2.32 is a schematic MSI for a high moisture food plotted to include the full range of moisture content from normal to dry. This kind of plot is not very useful because the data of greatest interest—those in the low moisture region—are not depicted in sufficient detail. Omission of the high moisture region and expansion of the low moisture region, as is normal practice, yields an MSI that is much more useful (Figure 2.33).

Moisture sorption isotherms exhibit a variety of shapes, many of which are amenable to at least qualitative interpretation. As an example, the sorption isotherms associated with several substances that have MSIs of markedly different shapes are shown in Figure 2.34. These are resorption (or adsorption) isotherms prepared by adding water to previously dried samples. Desorption isotherms are also common. The correspondence of adsorption and desorption isotherms will be discussed in Section 2.11.3. Isotherms with a sigmoidal shape are characteristic of most foods. However,

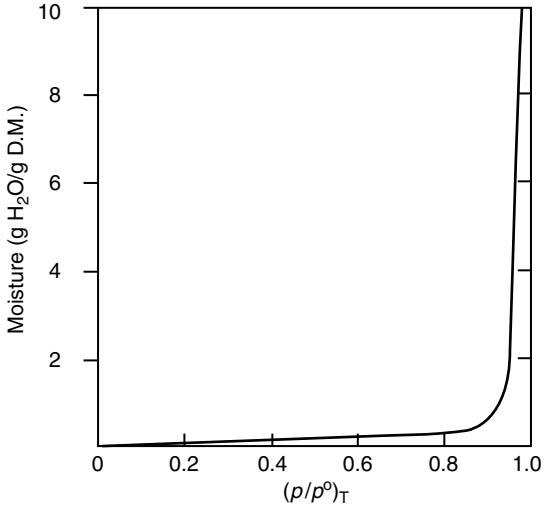

FIGURE 2.32 Schematic MSI encompassing a broad range of moisture contents.

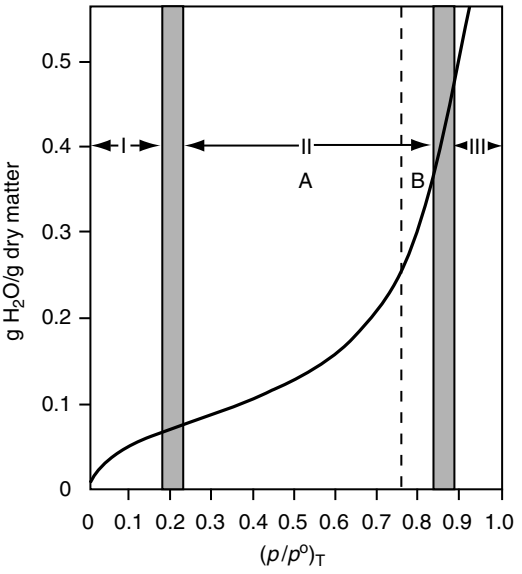

FIGURE 2.33 Generalized MSI for the low moisture segment of a food (20°C).

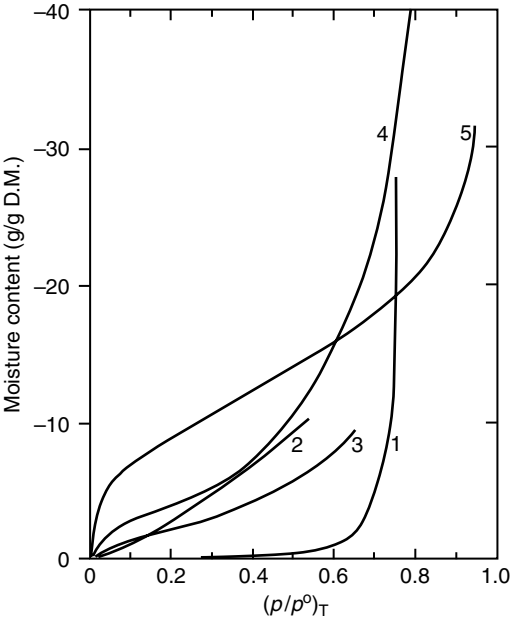

FIGURE 2.34 Resorption isotherms for various foods and biological substances. Temperature 20°C, except for number 1, which is 40°C. (1) confection (major component powdered sucrose), (2) spray dried chicory extract, (3) roasted Columbian coffee, (4) pig pancreas extract powder, and (5) native rice starch. (From van den Berg, C. and S. Bruin (1981) In *Water Activity: Influences on Food Quality* (L.B. Rockland and G.F. Stewart, Eds.), Academic Press: New York, pp. 1–61.)

foods (such as fruits, confections, and coffee extract) that contain large amounts of sugar and other small soluble molecules, and are not rich in sparingly soluble hydrophilic polymeric materials, may exhibit a J-type isotherm shown as curve 1 in Figure 2.34. The shapes and positions of the isotherms are determined by several factors including sample composition (including molecular

weight distribution and hydrophilic/hydrophobic characteristics of solutes), physical structure of the sample (e.g., crystalline or amorphous), sample pretreatments, temperature, and methodology.

Many attempts have been made to model MSIs, but success in achieving good conformance of a model to the full range of actual data for an MSI has been difficult. The oldest and best-known model is that of Brunauer, Emmett, and Teller (BET), derived for nonpolar gas sorption [83], but applied to aqueous systems with some redefinition of key terms. One of the better models is that developed by Guggenheim [84], Anderson [85], and De Boer [86], which is referred to as the GAB model. In food systems, it must be borne in mind that both models, though helpful, provide essentially empirical fits. The underlying models have parameters that are descriptive of much simpler systems. As an aid to interpreting moisture isotherms it is sometimes appropriate to divide them conceptually into zones as indicated in Figure 2.33. As water is added (resorption), the sample composition moves gradually from zone I (dry) to zone III (high moisture) and the properties of water associated with each zone differ significantly. These characteristic properties are described next and are summarized in Table 2.10. It should be realized that, even within a zone, water is freely exchanging and so the average properties of water within a zone depend upon the exact extent of population of the potential sites within the zone. The properties of water within different zones, however, are sufficiently different such that, on the commencement of populating a higher zone, even given easy exchange of water between zones, clear populations with different average properties corresponding to the separate zones can be seen.

Water present in amounts up to the zone I boundary limit of the isotherm can be considered most strongly sorbed and least mobile. This water is probably associated with accessible polar sites by water–ion or water–dipole interactions. It remains unfrozen at $-40°C$, it does not act as a solvent, and it is not present in sufficient amount to have a plasticizing effect on the solid. It behaves simply as a part of the solid.

The high-moisture end of zone I (boundary of zones I and II) corresponds to the "BET monolayer" moisture content of the food. Thus, the BET monolayer value can be thought of as corresponding approximately to the amount of water needed to form a monolayer over only the readily accessible, highly polar groups of the dry matter. In the case of starch, this amounts to one HOH per anhydroglucose unit. Zone I water is an amount corresponding to just a tiny fraction of the total water content in a high moisture food material. This amount of water clearly is less than the potential "sorption sites" represented by all of the polar or other active groups of the solute molecules. Additional water added in an amount not exceeding the limit set by the zone II boundary can be considered to populate those first-layer sites that are still available. This second water population, which probably associates with neighboring water molecules in this first layer and solute molecules primarily by hydrogen bonding, is slightly less mobile than bulk water and most of it remains unfrozen at $-40°C$. Moisture added in the vicinity of the low moisture end of zone II exerts a significant plasticizing action on solutes, lowers their glass transition temperatures, and causes incipient swelling of the solid matrix. Exchange of all water molecules is enhanced, but two populations become evident in relaxation spectroscopy experiments. This action, coupled with the beginning of the solution process, leads to acceleration in the rate of most reactions due to increasing interaction and accessibility. The amount of water that fully occupies zones I and II usually constitutes $<5\%$ of the water in a high moisture food material. Note that individual zones are defined only for water contents below that of their upper zone boundaries, since at this water concentration a new population begins to emerge. This initiates exchange processes that allow, at higher water contents, water molecules to exchange between separate populations. It is also a consequence of the influence of a more swollen, higher water content environment on access to potential interactive sites that were sterically or otherwise restricted in the early stages of sorption from being completely dry. Though now exchangeable, identifiable fractions of the water can be considered to share the characteristics of either zone I or zone II. Quantification of zone populations is a challenge, with perhaps the best estimates coming from relaxational spectroscopy. Lillford et al. [87] showed that relaxation spectroscopy in foods exhibited a complex decay curve that could be interpreted in

TABLE 2.10
Protein Hydration Levels

	Increasing Water Content in System					
	Constitutional Water[a]		Hydration Shell (≤3 Å from Surface)		Bulk-Phase Water	
Property					**Free[b]**	**Entrapped[c]**
Relative vapor pressure (p/p^o)	<0.02 p/p^o	0.02–0.2 p/p^o	0.2–0.75 p/p^o	0.75–0.85 p/p^o	>0.85 p/p^o	>0.85 p/p^o
Isotherm "zone"[d]	Extreme left, zone I	Zone I	Zone IIA	Zone IIB	Zone III	Zone III
Mol H_2O/mol dry protein	<8	8–56	56–200	200–300	>300	>300
g H_2O/g dry protein (h)	<0.01	0.01–0.07	0.07–0.25	0.25–0.58	>0.58	>0.58
Weight percent based on lysozyme (%)	1	1–6.5	6.5–20	20–27.5	>27.5	>27.5
Water characteristics: structure	Critical part of native protein structure	Water interacts primarily with charged groups (~2HOH/group). At 0.07 h transition in surface water organization; appearance of clusters associated with completion of charged group hydration	Water interacts primarily with polar surface groups (~1 HOH/polar site). Water clusters center on charged polar sites. Clusters fluctuate in size and arrangement. At 0.15 h long range connectivity of surface water is achieved	At 0.25 h water starts to condense on to weakly interacting unfilled protein surface patches. At 0.38 h "monolayer" of water covers the entire surface of the protein. Distinct water phase begins to appear, location of glass–rubber transition		
Water characteristics: thermodynamic transfer properties[e]						
ΔG (kJ/mol)	$>\lvert -6\rvert$	−6	−0.8	Close to bulk		
ΔH (kJ/mol)	$>\lvert -17\rvert$	−70	−2.1	Close to bulk		

Residence time (approximate mobility)	10^{-2}–10^{-8} s	$<10^{-8}$ s	$<10^{-9}$ s	10^{-9}–10^{-11} s	10^{-11}–10^{-12} s	10^{-11}–10^{-12} s
Freezability	Unfreezable	Unfreezable	Unfreezable	Unfreezable	Normal	Normal
Solvent power	None	None	Slight	Moderate	Normal	Normal
Protein characteristics: structure	Folded state, stable	Amorphous regions begin to be plasticized by water	Further plasticization of amorphous regions			
Protein characteristics: mobility (reflected in enzyme activity)	Enzyme activity negligible	Enzyme activity negligible	Proton exchange increases from 1/1000 at 0.04 h to full solution rater at 0.15 h. Some enzymes develop activity between 0.1 and 0.15 h	At 0.38 h lysozyme activity is 0.1 than in dilute solution	Maximum activity	Maximum activity

[a] Water molecules that occupy specific locations in the interior of the solute macromolecule.
[b] Macroscopic flow physically unconstrained by a macromolecular matrix.
[c] Macroscopic flow physically constrained by a macromolecular matrix.
[d] See Figure 2.33.
[e] Partial molar values for transfer of water from bulk phase to hydration shell.

Note: Constitutional water is assumed to be present in the dry protein at the onset of the hydration process. Water is first absorbed at sites of ionized carboxylic and amino side chains, with about 40 mol water/mol lysozyme associating in this manner. Further water absorption results in gradual hydration of less attractive sites, mainly amide carbonyl groups of the protein backbone. At 0.38 h monolayer coverage is achieved through water associating with those surface sites that are still less attractive. At this stage in hydration of the protein, there is, on average, 1 HOH/20 $Å^2$ of protein surface. At water content above 0.58 h the protein is considered fully hydrated.

Source: Data, largely on lysozyme, from Franks, F. (1988) In *Characteristics of Proteins* (F. Franks, Ed.), Humana Press: Clifton, NJ, pp. 127–154; Lounnas, V. and B.M. Pettitt (1994) *Proteins: Struc. Func. Genet.* **18**: 133–147; Rupley, J.A. and G. Careri (1991) *Adv. Protein Chem.* **41**: 37–172; Otting, G., et al. (1991) *Science* **254**: 974–980; and Lounnas, V. and B.M. Pettitt (1994) *Proteins: Struct. Func. Genet.* **18**: 148–160.

terms of water populations and exchange between populations. Hills and coworkers [88–91] further expanded upon this approach to quantify and characterize the changing water populations in different environments in foods, and in model systems, in particular starch-based systems. Schmidt [4] provides a detailed discussion of such studies. The results show greater complexity than the simple three-zone model, but confirm that this model provides a simple framework upon which to build an understanding.

As water content increases further, in the vicinity of the junction of zones II and III, the amount of water is sufficient to complete a true monolayer hydration shell for individual macromolecules such as globular proteins, and is also sufficient to lower the glass transition temperature of the hydrated macromolecules so that sample temperature and T_g are equal. A third separately detectable population enters the picture. Further addition of water (zone III) causes a glass–rubber transition in samples containing glassy regions, as evidenced by a very large decrease in viscosity, consequent upon a large increase in molecular mobility, and accompanied by commensurate increases in the rates of many reactions. Only at and beyond this water content at which the third population begins to appear can added water be frozen. Also, zone III water is available as a solvent and readily supports the growth of microorganisms. At water contents in excess of the lower zone III boundary, the additional water behaves as bulk-phase water (Table 2.10). Its addition to the system does not alter the properties of existing solutes, though as before all water molecules are freely exchanging. The zones are defined more as the populations of water molecules involved in particular classes of interaction with the solute. The relaxation spectroscopy studies show that as the water content increases, the various exchangeable populations may change in size. The water fraction at which a new population just begins to appear does not necessarily define the maximum size of the prior population at higher total water content.

In gels or cellular systems, bulk-phase water is physically entrapped so that macroscopic flow is impeded. However, in all other aspects this water has properties similar to those of water in a dilute salt solution. This is reasonable, since a typical water molecule, while occupying zone III, is "insulated" from the effects of solute molecules by several layers of zones I and II water molecules. The bulk-phase water population of zone III, either entrapped or free, usually constitutes more than 95% of the total water in a high moisture food, a fact that is not evident from Figure 2.33.

The important effects that such differences in water properties have on the stability of foods are discussed in a later section. At this point, suffice it to say that the most mobile water fraction existing in any food sample frequently governs stability.

2.11.2 Temperature Dependence

As mentioned earlier, RVP is temperature dependent, thus MSIs must also exhibit temperature dependence. An example of such temperature dependence involving potato slices is shown in Figure 2.35. At any given moisture content, food $(p/p^o)_T$ increases with increasing temperature, frequently in conformity with the Clausius–Clapeyron equation, though this conformity does not itself indicate true equilibrium.

2.11.3 Hysteresis

The foregoing discussion has indicated that MSIs may be obtained using either adsorption or desorption protocols. However, an additional complication to our discussion of MSIs is that a MSI prepared by the addition of water (resorption) to a dry sample will not necessarily be superimposable on an isotherm prepared by desorption. This lack of superimposability is referred to as "hysteresis" and a schematic example is shown in Figure 2.36. Typically, at any given $(p/p^o)_T$, the water content of the sample will be greater during desorption than during resorption. It has been found that MSIs of polymers, glasses of low molecular weight compounds, and many foods exhibit such sorption hysteresis [52,92].

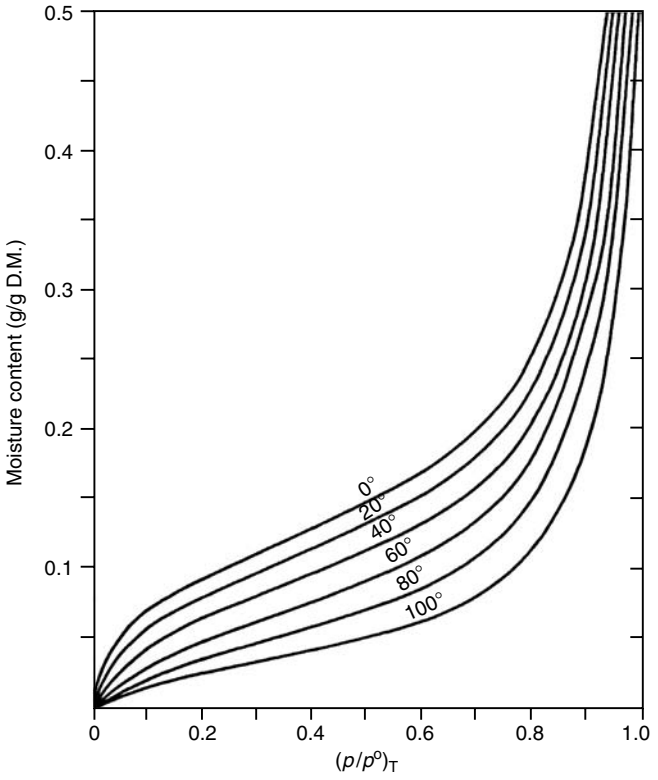

FIGURE 2.35 Moisture desorption isotherms for potatoes at various temperatures. (Redrawn from Gorling, P. (1958) In *Fundamental Aspects of the Dehydration of Foodstuffs*. Society of Chemical Industry: London, pp. 42–53.)

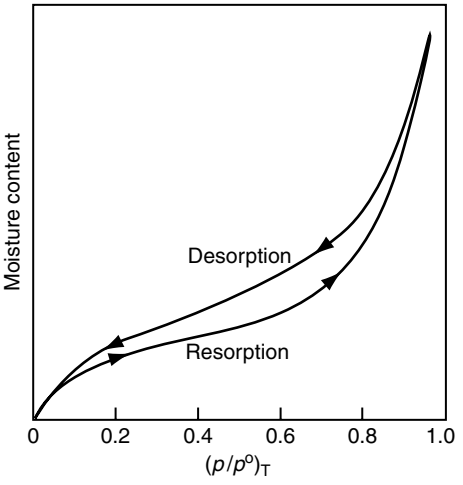

FIGURE 2.36 Hysteresis of a MSI.

The magnitude of hysteresis, the shape of the curves, and the inception and termination points of the hysteresis loop can vary considerably depending on factors such as nature of the food, the physical changes it undergoes when water is removed or added, temperature, the rate of desorption, and the degree of water removal during desorption [92]. The effect of temperature is pronounced:

hysteresis is often not detectable at high temperatures (~80°C) and generally becomes increasingly evident as the temperature is lowered.

Several largely qualitative theories have been advanced to explain sorption hysteresis [52,92]. These theories involve factors such as swelling phenomena, metastable local domains, chemisorption, phase transitions, capillary phenomena, and the fact that nonequilibrium states become increasingly persistent as the temperature is lowered. A definitive explanation (or explanations) of sorption hysteresis has yet to be formulated.

Sorption hysteresis is more than a laboratory curiosity. Labuza et al. [93] have conclusively demonstrated that lipid oxidation in strained meat from chicken and pork at $(p/p^o)_T$ values in the range 0.75–0.84 proceeds much more rapidly if the samples are adjusted to the desired $(p/p^o)_T$ value by desorption rather than resorption. The desorption samples, as already noted, contain more water at a given $(p/p^o)_T$ than the resorption samples. This would cause the high moisture sample to have a lower viscosity, which in turn would cause greater catalyst mobility, greater exposure of catalytic sites because of the swollen matrix, and somewhat greater oxygen diffusivity than in the lower moisture (resorption) sample. In another study, Labuza et al. [94] found that the $(p/p^o)_T$ needed to stop the growth of several microorganisms is significantly lower if the product is prepared by desorption rather than resorption. The existence of hysteresis is yet further compelling evidence that, as commonly determined, sorption isotherms define steady-state systems (assuming that sufficient time has been allowed for the system to attain steady state) rather than true equilibrium systems.

By now it should be very clear that MSIs are highly product specific, that the MSI for a given product can be changed significantly by the manner in which the product is prepared, and that these points are of practical importance. Further discussion on the measurement and utility of MSIs can be found in References 48 and 95–98 and a compilation of typical MSIs is to be found in Reference 99.

2.11.4 Hydration Sequence of a Protein

It is instructive to consider further water absorption by a dry food component and the location and properties of water at each state of the process. Lyoszyme is chosen for this exercise because proteins are of major importance in foods and because they contain all of the major types of functional group important to hydration. Even "dry" lysozyme contains some constitutional water, which is an integral part of the structure. This amounts to around 8 mol water/g dry protein. On exposure to increasing levels of RVP, water is first adsorbed at the sites of ionized, carboxylic acid, and amino side chains. This requires about 40 mol water/mol dry lysozyme and corresponds approximately to the water content at the BET monolayer, the junction of zones I and IIA, at a RVP around 0.2. Further increase in RVP, to around 0.25 (end of zone IIA) leads to sorption at less active sites such as amide carbonyls, and continued sorption to RVP 0.75 (end of zone IIB) results in full surface coverage with water content 0.38. At this point (the junction of zones IIB and III), all available surface sites are considered covered [100]. Beyond this RVP (zone III) the water corresponds to multilayer (bulk) water. Enzyme activity is first seen above the "BET monolayer" coverage, and maximum activity is reached at the full surface coverage point. These observations help illustrate the value of the zonal description of the MSI in categorizing hydration effects. It must be remembered, however, that at any particular water content, all water molecules are freely exchangeable between regions, leading to a continuum of behaviors as water content increases.

2.12 RELATIVE VAPOR PRESSURE AND FOOD STABILITY

Historically, it has often been demonstrated that food stability and $(p/p^o)_T$ are closely related in many situations. The data of Figures 2.20, 2.37 and Table 2.11 provide examples of these relationships. Shown in Table 2.11 are various common microorganisms and the range of RVP permitting their growth [101]. Also shown in this table are common foods categorized according to their RVP.

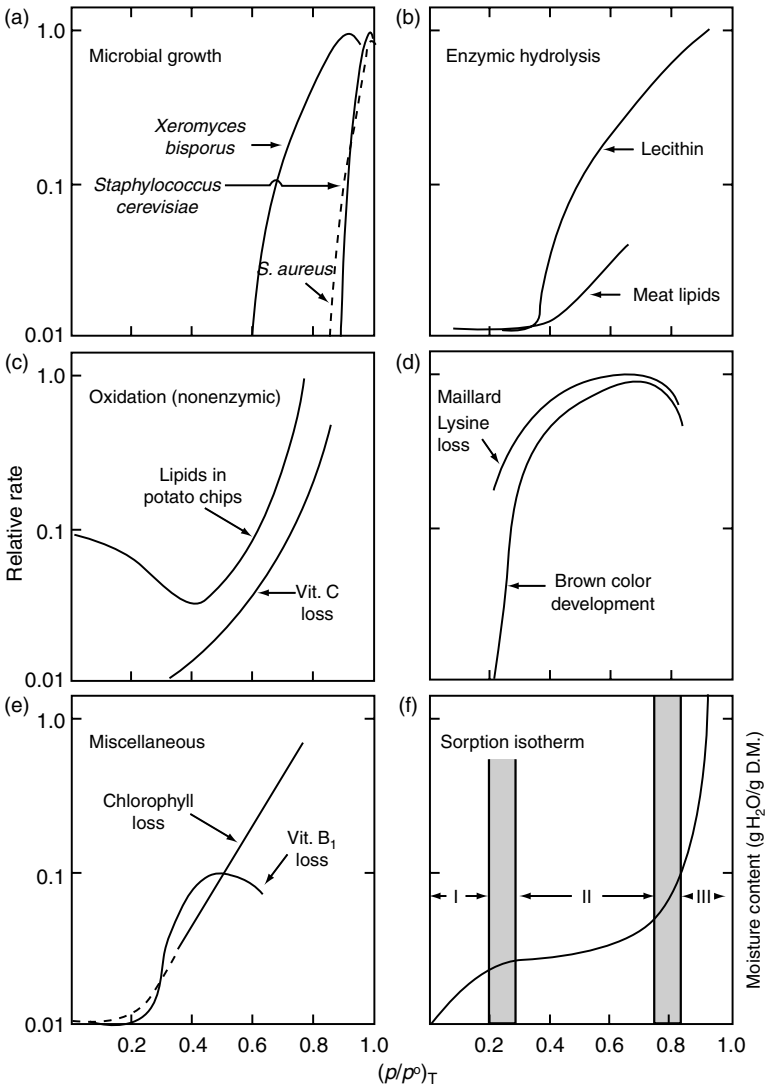

FIGURE 2.37 Relationships among relative water vapor pressure, food stability, and sorption isotherms. (a) Microbial growth vs. $(p/p^{o})_T$, (b) enzymic hydrolysis vs. $(p/p^{o})_T$, (c) oxidation (nonenzymic) vs. $(p/p^{o})_T$, (d) Maillard browning vs. $(p/p^{o})_T$, (e) miscellaneous reaction rates vs. $(p/p^{o})_T$, and (f) water content vs. $(p/p^{o})_T$. All ordinates are "relative rate" except for F. Data from various sources.

Data in Figure 2.37 are typical relationships between reaction rate and $(p/p^{o})_T$ in the temperature range 25–45°C. For comparative purposes a typical isotherm is also shown in Figure 2.37f. It is important to remember that the exact reaction rates and the positions and shapes of the curves in Figure 2.37 can be altered by sample composition, physical state and structure of the sample, composition of the atmosphere (especially oxygen), temperature, and by hysteresis effects. The reader is also cautioned that this empirical relationship is between a thermodynamic parameter and a kinetic parameter, and that there is no intrinsic theoretical reason why such parameters should correlate, since thermodynamics deals with equilibrium positions, and kinetics deals with rates. Thermodynamics is predictable and kinetics is empirical.

The unusual relationship between the rate of lipid oxidation and $(p/p^{o})_T$ at very low values of $(p/p^{o})_T$ deserves comment (Figure 2.37). Starting at the extreme left of the isotherm, added

TABLE 2.11
Potential for Growth of Microorganisms in Food at Different Relative Vapor Pressures

Range of p/p^o	Microorganisms Generally Inhibited by Lowest p/p^o of the Range	Foods Generally within this Range of p/p^o
1.00–0.95	*Pseudomonas, Escherischia Proteus, Shigella, Klebsiella, Bacillus, Clostridium perfringens,* some yeasts	Highly perishable (fresh) foods, canned fruits, vegetables, meat, fish, and milk; cooked sausages and breads; foods containing up to 7% (w/w) sodium chloride or 40% sucrose
0.95–0.91	*Salmonella*, *Vibrio parahaemolyticus*, *C. Botulinum*, *Serratia*, *Lactobacillus*, some molds, yeasts (*Rhodotorula*, *Pichia*)	Some cheeses (Cheddar, Swiss, Muenster, Provolone), cured meats (ham), some fruit juice concentrates, foods containing up to 12% (w/w) sodium chloride or 55% sucrose
0.91–0.87	Many yeasts (*Candida, Torulopsis, Hansenula, Micrococcus*)	Fermented sausages (salami), sponge cakes, dry cheeses, margarine, foods containing up to 15% (w/w) sodium chloride or saturated (65%) sucrose
0.87–0.80	Most molds (mycotoxigenic penicillia), *Staphylococcus aureus*, most *Saccharomyces* (*bailii*) spp., *Debaryomyces*	Most fruit juice concentrates, sweetened condensed milk, chocolate syrup, maple and fruit syrups; flour, rice, pulses of 15–17% moisture content; fruit cake; country style ham, fondants
0.80–0.75	Most halophilic bacteria, mycotoxigenic aspergilli	Jam, marmalade, marzipan, glace fruits, some marshmallows
0.75–0.65	Xerophilic molds (*Aspergillus chevalieri, A. candidus, Wallemia sebi*) *Saccharomyces bisporus*	Rolled oats of 10% moisture content; grained nougats, fudge, marshmallows, jelly, molasses, raw cane sugar, some dried fruits, nuts
0.65–0.60	Osmophilic yeasts (*Saccharomyces rouxii*), few molds (*Aspergillus echinulatus, Monascus bisporus*)	Dried fruits of 15–20% moisture content, toffees and caramels, honey
0.60–0.50	No microbial proliferation	Pasta of 12% moisture content, spices of 10% moisture content
0.50–0.40	No microbial proliferation	Whole egg powder of 5% moisture content
0.40–0.30	No microbial proliferation	Cookies, crackers, bread crusts, and so forth of 3–5% moisture content
0.30–0.20	No microbial proliferation	Whole milk powder of 2–3% moisture content; dried vegetables of 5% moisture content; corn flakes of 5% moisture content, country style cookies, crackers

Source: Beuchat, L.R. (1981) *Cereal Foods World* **26**: 345–349.

water decreases the rate of oxidation until a water content equivalent to the BET monolayer value is attained. Clearly, overdrying of samples subject to oxidation will result in less than optimal stability. Karel and Yong [102] have offered the following interpretative suggestions regarding this behavior. The first water added to a very dry sample is believed to bind hydroperoxides, interfering with their decomposition, and thereby hindering the progress of oxidation. In addition, this water hydrates metal ions that catalyze oxidation, apparently reducing their effectiveness.

Addition of water beyond the boundary of zones I and II (Figure 2.37) results in increased rates of oxidation. Karel and Yong suggested that water added in this region of the isotherm accelerates

oxidation by increasing the solubility of oxygen and by allowing macromolecules to swell, thereby exposing more catalytic sites. At still greater $(p/p^o)_T$ values ($>\sim 0.80$), the added water may retard rates of oxidation, and the suggested explanation is that dilution of catalysts reduces their effectiveness.

It should be noted that the curves for the Maillard reaction, vitamin B_1 degradation, and microbial growth all exhibit rate maxima at intermediate to high $(p/p^o)_T$ values (Figure 2.37). Two possibilities have been advanced to account for the decline in reaction rate that sometimes accompanies increases in RVP in foods having moderate to high moisture contents:

1. For those reactions in which water is a product, an increase in water content can result in product inhibition.
2. When the water content of the sample is such that the solubility, accessibility (surfaces of macromolecules), and mobility of rate-enhancing constituents are no longer rate limiting, further addition of water serves only to dilute rate-enhancing constituents and decrease the reaction rate.

Since the BET monolayer value of a food frequently provides a good first estimate of the water content providing maximum stability of a dry product, knowledge of this value is of considerable practical importance. Determining the BET monolayer value for a specific food can be done with moderate ease if data for the low-moisture end of the MSI are available. One can then use the BET equation developed by Brunauer et al. [83] to compute the monolayer value:

$$\frac{a_w}{m(1-a_w)} = \frac{1}{m_1 c} + \frac{c-1}{m_1 c} a_w \tag{2.12}$$

where a_w is water activity, m is moisture content (g H_2O/g dry matter), m_1 is the BET monolayer value and c is a constant. In practice $(p/p^o)_T$ values are used in Equation 2.12 rather than a_w values.

From this equation it is apparent that a plot of $a_w/m\,(1-a_w)$ vs. a_w, known as a BET plot, should yield a straight line. An example, for native potato starch with a_w replaced by $(p/p^o)_T$ is shown in Figure 2.38. The linear relationship, as is generally acknowledged, begins to deteriorate at $(p/p^o)_T$ values greater than about 0.35.

The BET monolayer value can be calculated as follows:

$$\text{Monolayer value} = m_1 = 1/((y \text{ intercept}) + (\text{slope}))$$

From Figure 2.38, the y intercept is 0.6. Calculation of the slope yields a value of 10.7. Thus:

$$m_1 = 1/(0.6 + 10.7) = 0.088 \text{ g } H_2O/\text{g dry matter}$$

In this particular example, the BET monolayer value corresponds to a $(p/p^o)_T$ of 0.2. The GAB equation yields a similar monolayer value.

In addition to chemical reactions and microbial growth, $(p/p^o)_T$ also influences the texture of dry and semidry foods. For example, suitably low RVPs are necessary if crispness of crackers, popped corn, and potato chips is to be retained; if caking of granulated sugar, dry milk, and instant coffee is to be avoided; and if stickiness of hard candy is to be prevented. The maximum $(p/p^o)_T$ that can be tolerated in dry materials without incurring loss of desirable properties ranges from 0.35 to 0.5, depending on the product [103]. Furthermore, suitably high $(p/p^o)_T$ values of soft-textured foods are needed to avoid undesirable hardness.

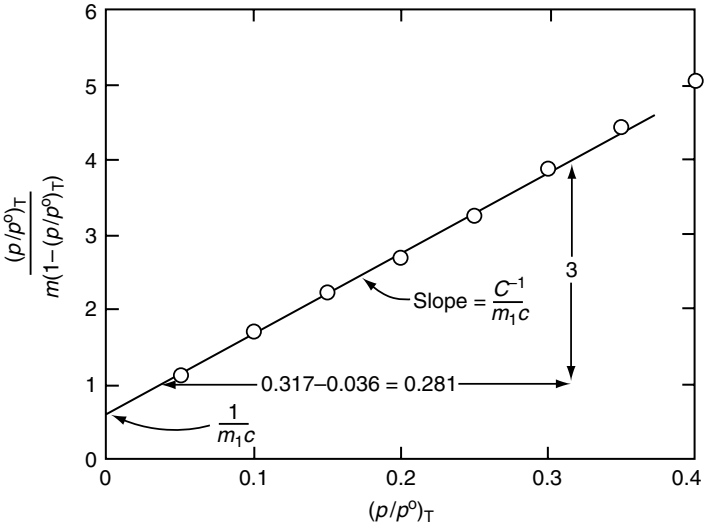

FIGURE 2.38 BET plot for native potato starch (resorption data, 20°C). (Data from van den Berg, C. (1981) *Vapour Sorption Equilibria and Other Water–Starch Interactions: A Physico-Chemical Approach*. Wageningen Agricultural University: Wageningen, The Netherlands.)

2.13 COMPARISONS

2.13.1 The Interrelationships Between the RVP, Mm, and MSI Approaches to Understanding the Role of Water in Foods

The equilibrium freezing temperature of a system provides a measure of its a_w, since at the freezing point the a_w of the system is identical to that of pure water ice at that same temperature. Bearing this in mind, we can construct an annotated state diagram (Figure 2.39) to map out the interrelationships between the RVP, Mm, and MSI approaches to understanding the roles of water in foods. The Mm approach has already been extensively discussed using appropriate state diagrams. In Figure 2.39, the area where RVP is most utilized is the top left. Considering systems of RVP = 0.8. These lie to the left of the line representing the composition of a system with a T_m^L of −22°C, the temperature where the RVP of ice is 0.8. The region of primary applicability of RVP to microbial stability is represented by the hatched box. This is far removed from the T_d and T_g lines, indicating that Mm of both solutes and solvent is sufficient to allow rapid rearrangement and adjustment to a long-term steady-state condition at a constant temperature. At lower RVP, corresponding to systems with hypothetical equilibrium freezing temperatures below −30°C, the corresponding RVP is less than 0.75. In such systems, solute mobilities are reduced, and attaining long-term steady state is more challenging. At RVP below 0.6 the hypothetical T_m^L would be around −52°C. Such systems are difficult to equilibrate, and measurement of a meaningful steady-state RVP is difficult, if not impossible.

In MSI, we tend to utilize both sorption and desorption. Consider first sorption. Plots from the right side of the state diagram represent sorption on to a dry product, with the right axis representing the dry product. The condition of the dry product defines whether line T_g or line T_m^s is appropriate to describe the state boundary as moisture content increases. Frequently partial crystallization has occurred within the dry product, such that portions of the product are appropriately considered in terms of each of these lines defining a change in physical state. Entry to the more mobile, fluid states occurs with initially low mobilities, and the processes of change may be slow. Water enters the product first at the surfaces, resulting in mobility gradients; as with a higher moisture content, surface mobility

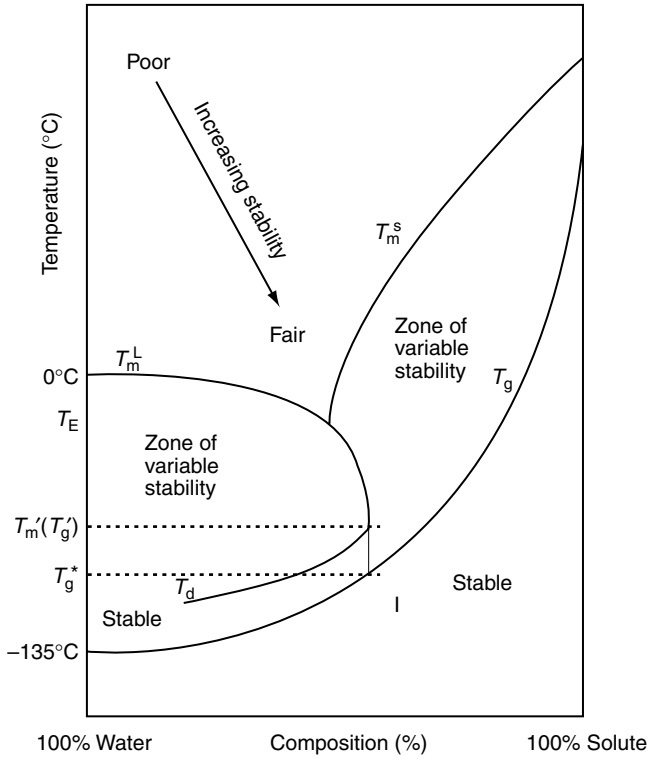

FIGURE 2.39 State diagram of a binary system showing potential stabilities in different zones.

is enhanced (and surface T_g is reduced). For desorption, the composition of samples is moving from left to right on the diagram, and a more fluid system is losing mobility as desorption progresses. Hence, rapid desorption will tend to lead to less solute crystallization than will slow desorption.

2.14 CONCLUSION

Each of the approaches to product stability described has best applicability under certain, constrained conditions. It is appropriate, therefore, to utilize all approaches in order to better appreciate the role of water in foods, and the potential mechanisms and routes through which water, and water content, can influence product stability. Both Schmidt [4] and Sherwin and Labuza [104] in recent articles have provided useful discussion of the relative importance of each approach under a range of different conditions. Water plays a critical role in the chemical and physical processes within foods. While an apparently simple molecule, it is clear that the complex nature of the hydrogen bonding networks of water, and between water and solutes, in addition to other influences upon the intermolecular arrangements of water are essential for the functioning of biological systems, and are key to the properties of foods.

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3 Carbohydrates

James N. BeMiller and Kerry C. Huber

CONTENTS

Carbohydrates comprise more than 90% of the dry matter of plants. As a result, they are abundant, widely available, and inexpensive. Carbohydrates are common components of foods, both as natural components and as added ingredients. Both the quantities consumed and the variety of products in which they are found are large. They have many different molecular structures, sizes, and shapes, exhibit a variety of chemical and physical properties, and differ in their physiological effects on the human body. They are amenable to both chemical and biochemical modification, and both modifications are employed commercially in improving their properties and extending their use.

Starch, lactose, and sucrose are digested by normal humans, and they, along with D-glucose and D-fructose, are human energy sources, providing 70–80% of the calories in the human diet worldwide. In the United States, they supply less than that percentage with widely varying amounts from individual to individual.

The term carbohydrate suggests a general elemental composition, namely, $C_x(H_2O)_y$, which signifies molecules containing carbon atoms along with hydrogen and oxygen atoms in the same ratio as they occur in water. However, the great majority of natural carbohydrate compounds produced by living organisms do not have this simple empirical formula. Rather, most natural carbohydrates are in the form of oligomers (oligosaccharides) or polymers (polysaccharides) of simple and modified sugars. The source of low-molecular-weight carbohydrates is often the depolymerized natural polymers. However, this chapter begins with a presentation of simple sugars and builds from there to larger and more complex structures.

3.1 MONOSACCHARIDES

Carbohydrates contain chiral carbon atoms. A chiral carbon atom is one that can exist in two different spatial arrangements (configurations). Chiral carbon atoms have four different groups attached to them. The two different arrangements of the four groups in space (configurations) are nonsuperimposable mirror images of each other (Figure 3.1). In other words, one is the reflection of the other that one would see in a mirror, with everything that is on the right in one configuration on the left in the other and vice versa.

D-Glucose, the most abundant carbohydrate and the most abundant organic compound (if all of its combined forms are considered), belongs to the class of carbohydrates called monosaccharides. Monosaccharides are carbohydrate molecules that cannot be broken down to simpler carbohydrate molecules by hydrolysis, so they are sometimes referred to as simple sugars. They are the monomeric

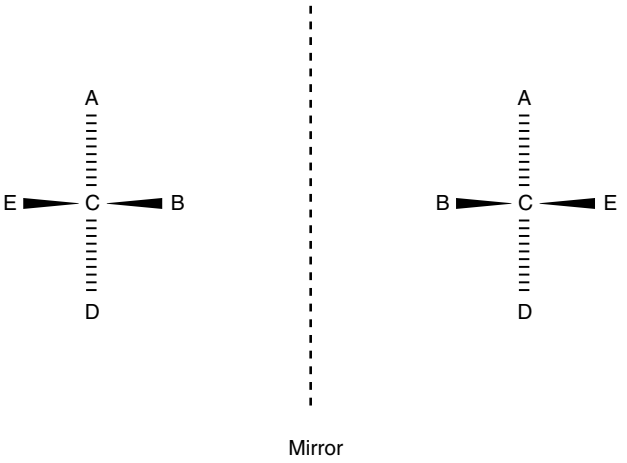

FIGURE 3.1 A chiral carbon atom. A, B, D, and E represent different atoms, functional groups, or other groups of atoms attached to the carbon atom C. Wedges indicate chemical bonds projecting outward from the plane of the page; dashes indicate chemical bonds projecting into or below the plane of the page.

TABLE 3.1
Classification of Monosaccharides

	Kind of Carbonyl Group	
Number of Carbon Atoms	**Aldehyde**	**Ketone**
3	Triose	Triulose
4	Tetrose	Tetrulose
5	Pentose	Pentulose
6	Hexose	Hexulose
7	Heptose	Heptulose
8	Octose	Octulose
9	Nonose	Nonulose

units that are joined together to form larger structures, namely, oligosaccharides and polysaccharides (see Sections 3.2 and 3.3), which can be converted into their constituent monosaccharides by hydrolysis.

D-Glucose is both a polyalcohol and an aldehyde. It is classified as an aldose, a designation for sugars containing an aldehyde group (Table 3.1). The suffix -ose signifies a sugar; the prefix ald- signifies an aldehyde group. When D-glucose is written in an open or vertical, straight-chain fashion (Figure 3.2) known as an acyclic structure, with the aldehyde group (carbon atom 1) at the top and the primary hydroxyl group (carbon atom 6) at the bottom, it is seen that all secondary hydroxyl groups are on carbon atoms 2, 3, 4, and 5, all of which have four different substituents attached to them and are, therefore, chiral. Naturally occurring glucose is designated as the D form; specifically, it is D-glucose. It has a molecular mirror image, termed L-glucose. Since each chiral carbon atom has a mirror image, there are 2^n arrangements for these atoms. Therefore, in a six-carbon aldose such as D-glucose (with its four chiral carbon atoms), there are 2^4 or 16 different arrangements of the carbon atoms containing secondary hydroxyl groups, allowing formation of 16 different six-carbon sugars with an aldehyde end. Eight of these belong to the D-series (Figure 3.3); eight are their mirror images and belong to the L-series. All sugars that have the hydroxyl group on the highest-numbered chiral carbon atom (C-5 in this case) positioned on the right-hand side are arbitrarily called D-sugars

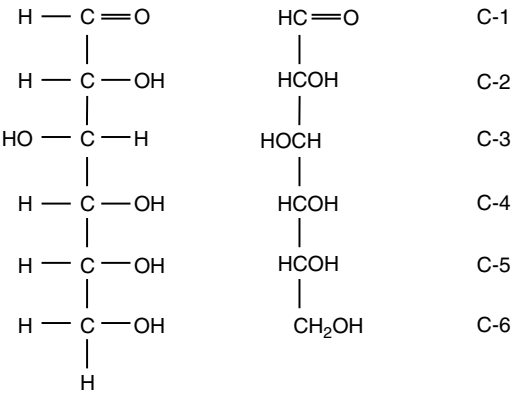

FIGURE 3.2 D-Glucose (open-chain or acyclic structure).

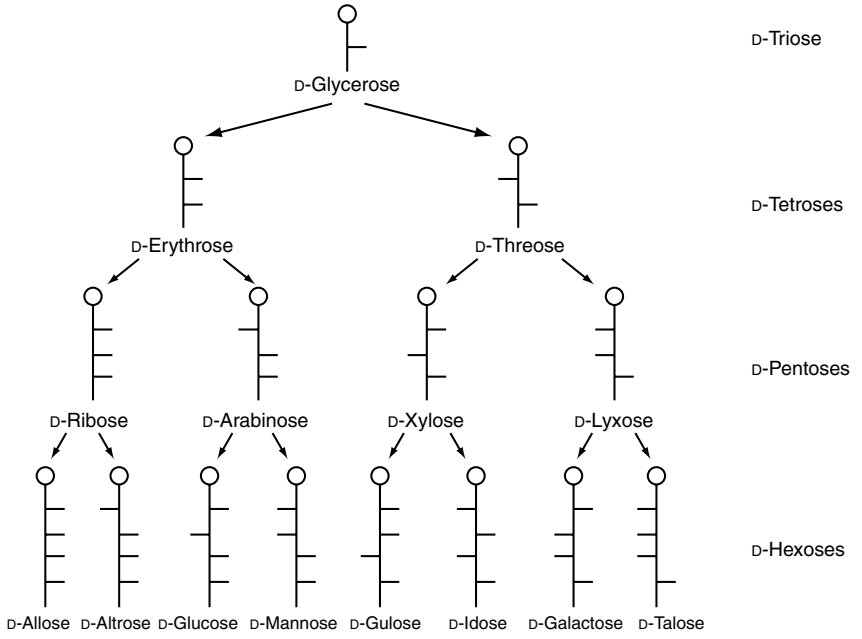

FIGURE 3.3 Rosanoff structure of the D-aldoses containing from 3 to 6 carbon atoms.

and all with a left-hand positioned hydroxyl group on the highest numbered chiral carbon atom are designated L-sugars. Two structures of D-glucose in its open-chain or acyclic form (called the Fischer projection) with the carbon atoms numbered in the conventional manner are given in Figure 3.2. In this convention, each horizontal bond projects outward from the plane of the page and each vertical bond projects into the plane of the page. (It is customary to omit the horizontal lines for covalent chemical bonds to the hydrogen atoms and hydroxyl groups as in the structure on the right.) Because the lowermost carbon atom is nonchiral, it is meaningless to designate the relative positions of the atoms and groups attached to it. Thus, it is written as $-CH_2OH$.

D-Glucose and all other sugars containing six carbon atoms are called hexoses, the group of aldoses present in nature in the greatest amount. The categorical names are often combined, a six carbon atom aldehyde sugar being termed an aldohexose.

There are two aldoses containing three carbon atoms. They are D-glyceraldehyde (D-glycerose) and L-glyceraldehyde (L-glycerose), each possessing but one chiral carbon atom. Aldoses with four

carbon atoms, the tetroses, have two chiral carbon atoms; aldoses with five carbon atoms, the pentoses, have three chiral carbon atoms and comprise the second most common group of aldoses. Extending the series above six carbon atoms gives heptoses, octoses, and nonoses, which is the practical limit of naturally occurring sugars. Development of the eight D-hexoses from D-glyceraldehyde is shown in Figure 3.3. In this figure, the circle represents the aldehyde group; the horizontal lines designate the location of each hydroxyl group on its chiral carbon atom, and at the bottoms of the vertical lines is the terminal, nonchiral, primary hydroxyl group ($-CH_2OH$). This shorthand way of indicating monosaccharide structures is called the Rosanoff method. Sugars whose names are in italics in Figure 3.3 are commonly found in plants, almost exclusively in combined forms, that is, in glycosides, oligosaccharides, and polysaccharides (see later). D-Glucose is the only free aldose usually present in natural foods, and that too only in small amounts.

L-Sugars are less numerous and less abundant in nature than are the D-forms, but nevertheless, have important biochemical roles. Two L-sugars found in foods are L-arabinose and L-galactose, both of which occur as units in carbohydrate polymers (polysaccharides).

In the other type of monosaccharide, the carbonyl function is a ketone group. These sugars are called ketoses. (The prefix ket- signifies the ketone group.) The suffix designating a ketose in systematic carbohydrate nomenclature is -ulose (Table 3.1). D-Fructose (systematically D-*arabino*-hexulose) is the prime example of this sugar group (Figure 3.4). It is one of the two monosaccharide units of the disaccharide sucrose (see Section 3.2.3) and makes up about 55% of common high-fructose syrup (HFS) and about 40% of honey. D-Fructose has only three chiral carbon atoms (C-3, C-4, and C-5). Thus, there are but 2^3 or eight ketohexoses. D-Fructose is the only commercial ketose and the only one found free in natural foods, but, like D-glucose, only in small amounts.

3.1.1 Monosaccharide Isomerization

Simple aldoses and ketoses containing the same number of carbon atoms are isomers of each other, that is, a hexose and a hexulose both have the empirical formula $C_6H_{12}O$ and can be interconverted by isomerization. Isomerization of monosaccharides involves both the carbonyl group and the adjacent hydroxyl group. By this reaction, an aldose is converted into another aldose (with the opposite configuration of C-2) and the corresponding ketose, and a ketose is converted into the corresponding two aldoses. Therefore, by isomerization, D-glucose, D-mannose, and D-fructose can be interconverted (Figure 3.5). Isomerization can be catalyzed by either a base or an enzyme.

3.1.2 Monosaccharide Ring Forms

Carbonyl groups of aldehydes are reactive and readily undergo nucleophilic attack by the oxygen atom of a hydroxyl group to produce a hemiacetal. The hydroxyl group of a hemiacetal can react further (by condensation) with a hydroxyl group of an alcohol to produce an acetal (Figure 3.6). The carbonyl group of a ketone reacts similarly.

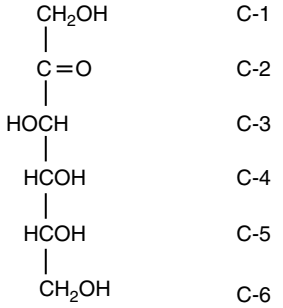

FIGURE 3.4 D-Fructose (open-chain or acyclic structure).

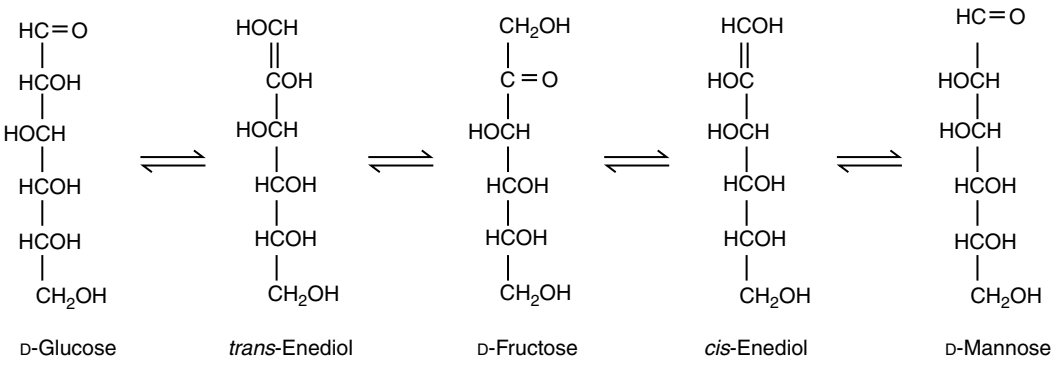

FIGURE 3.5 Interrelationship of D-glucose, D-mannose, and D-fructose via isomerization.

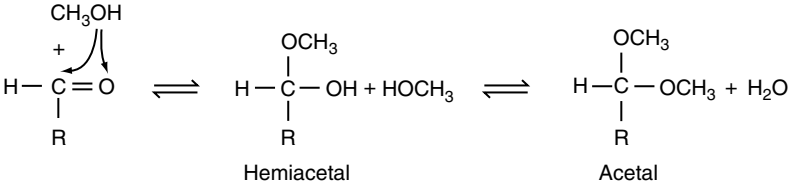

FIGURE 3.6 Formation of an acetal by reaction of an aldehyde with methanol.

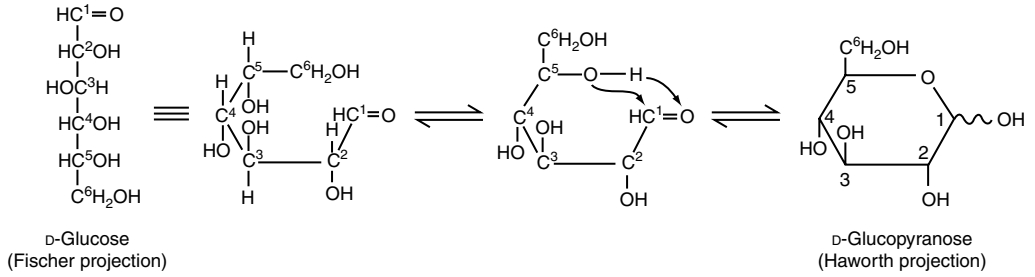

FIGURE 3.7 Formation of a pyranose hemiacetal ring from D-glucose.

Hemiacetal formation can occur within the same aldose or ketose sugar molecule, that is, the carbonyl group of a sugar molecule can react with one of its own hydroxyl groups, as illustrated in Figure 3.7 with D-glucose laid coiled on its side. The six-membered sugar ring that results from reaction of an aldehydo group with the hydroxyl group at C-5 is called a pyranose ring. Notice that, for the oxygen atom of the hydroxyl group at C-5 to react to form the ring, C-5 must rotate to bring its oxygen atom upward. This rotation brings the hydroxymethyl group (C-6) to a position above the ring. The representation of the D-glucopyranose ring used in Figure 3.7 is termed a Haworth projection.

Sugars also occur in five-membered (furanose) rings (Figure 3.8), but less frequently than they do in pyranose rings.

To avoid clutter in writing the ring structures, common conventions are adopted wherein ring carbon atoms are indicated by angles in the ring and hydrogen atoms attached to carbon atoms are eliminated altogether. A mixture of chiral (anomeric*) forms is indicated by a wavy line (Figure 3.9).

* The α and β ring forms of a sugar are known as anomers. The two anomers comprise an anomeric pair.

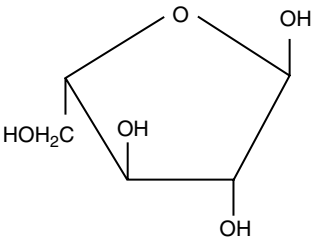

FIGURE 3.8 L-Arabinose in the furanose ring form and α-L-configuration.

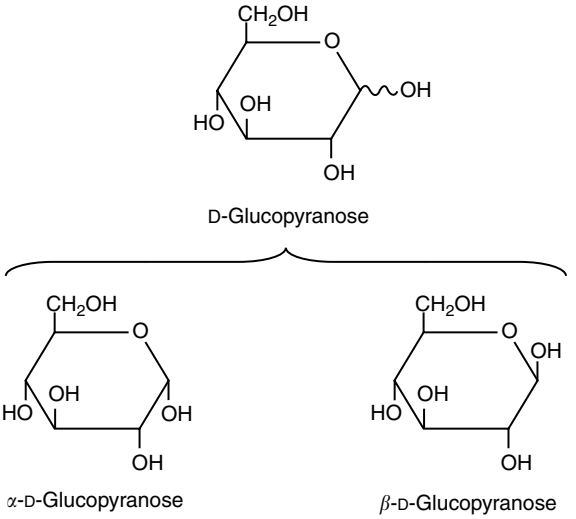

FIGURE 3.9 D-Glucopyranose as a mixture of two chiral forms.

When the carbon atom of the carbonyl group is involved in ring formation, leading to hemiacetal (pyranose or furanose ring) development, it becomes chiral. With D-sugars, the configuration that has the hydroxyl group located below the plane of the ring (in the Haworth projection) is the alpha form. For example, therefore, α-D-glucopyranose is D-glucose in the pyranose (six-membered) ring form with the configuration of the new chiral carbon atom, C-1, termed the anomeric carbon atom, alpha (below the plane of the ring). When the newly formed hydroxyl group at C-1 is above the plane of the ring (in the Haworth projection), it is in the beta position, and the structure is named β-D-glucopyranose. This designation holds for all D-sugars. For sugars in the L-series, the opposite is true, that is, the anomeric hydroxyl group is up in the alpha anomer and down in the beta anomer (see Figure 3.8). This is so because, for example, α-D-glucopyranose and α-L-glucopyranose are mirror images of one another.

However, pyranose rings are not flat with the attached groups sticking straight up and straight down as the Haworth representation suggests. Rather, they occur in a variety of shapes (conformations), most commonly in one of two chair conformations, so-called because they are shaped somewhat like a chair. In a chair conformation, one bond on each carbon atom does project either up or down from the ring; these are called axial bonds or axial positions. The other bond not involved in ring formation, is either up or down with respect to the axial bonds but, with respect to the ring, projects out around the perimeter in what is called an equatorial position (Figure 3.10).

Using β-D-glucopyranose as an example, C-2, C-3, C-5, and the ring oxygen atom remain in a plane, but C-4 is raised slightly above the plane and C-1 is positioned slightly below the plane as in Figures 3.10 and 3.11. This conformation is designated $^4C_1$. The notation C indicates that the

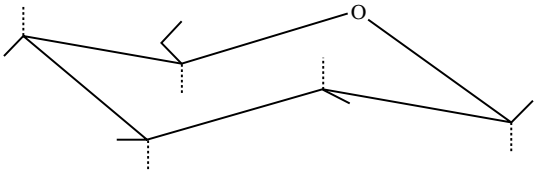

FIGURE 3.10 A pyranose ring showing the equatorial (solid line) and axial (dashed line) bond positions.

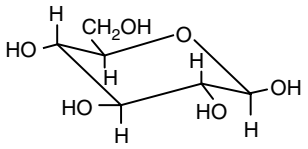

FIGURE 3.11 β-D-Glucopyranose in the $^4C_1$ conformation. All bulky groups are in equatorial positions and all hydrogen atoms in axial positions.

ring is chair-shaped; the superscript number indicates that C-4 is above the plane of the ring and the subscript number indicates that C-1 is below the plane. (There are two chair forms. The second, $^1C_4$, has all the axial and equatorial groups reversed.) The six-membered ring distorts the normal carbon and oxygen atom bond angles less than do rings of other sizes. The strain is further lessened when the bulky hydroxyl groups are separated maximally from each other by the ring conformation that arranges the greatest number of them in equatorial, rather than axial, positions. The equatorial position is energetically favored and rotation of carbon atoms takes place on their connecting bonds to swivel the bulky groups to equatorial positions as far as possible.

As noted, β-D-glucopyranose has all its hydroxyl groups in the equatorial arrangement, but each is either slightly above or slightly below the true equatorial position. In β-D-glucopyranose, the hydroxyl groups, all of which are in an equatorial position, alternate in an up-and-down arrangement, with that on C-1 positioned slightly up, that on C-2 slightly down, and continuing with an up-and-down arrangement. The bulky hydroxymethyl group, C-6 in hexoses, is almost always in a sterically free equatorial position. If β-D-glucopyranose were in a $^1C_4$ conformation, all the bulky groups would be axial. Being, a much higher energy form, little of D-glucopyranose exists in the $^1C_4$ conformation.

Six-membered sugar rings are then quite stable if bulky groups such as hydroxyl groups and the hydroxymethyl group are in equatorial positions. Thus, β-D-glucopyranose dissolves in water to give a rapidly equilibrating mixture containing the open-chain form and its five-, six-, and seven-membered ring forms. At room temperature, the six-membered (pyranose) ring forms predominate, followed by the five-membered (furanose) ring forms. The configuration of the anomeric carbon atom (C-1 of aldoses) of each ring may be alpha or beta. The equilibrium ratio of the ring forms varies with the sugar and the temperature. Examples of the distribution are given in Table 3.2.

The open-chain, aldehydo form constitutes only about 0.003% of the total forms; but because of rapid interconversion with the ring forms, a sugar can readily and rapidly react as if it where entirely in the free aldehyde form (Figure 3.12).

3.1.3 Glycosides

The hemiacetal form of sugars can react with an alcohol to produce a full acetal; the product is called a glycoside. In the laboratory, the reaction occurs under anhydrous conditions in the presence of the acid (as a catalyst) at elevated temperatures, but glycosides are most commonly made in nature, that is, in aqueous environments by enzyme-catalyzed reactions in pathways involving several intermediates. The acetal linkage at the anomeric carbon atom is indicated by the -ide suffix. In the case of D-glucose reacting with methanol, the product is mainly methyl α-D-glucopyranoside, with

TABLE 3.2
Equilibrium Distribution of Cyclic and Anomeric Forms of Monosaccharides

	Pyranose Ring Forms		Furanose Ring Forms	
Sugar	**α-**	**β-**	**α-**	**β-**
Glucose	36.2	63.8	0	0
Galactose	29	64	3	4
Mannose	68.8	31.2	0	0
Arabinose	60	35.5	2.5	0.5
Ribose	21.5	58.5	6.5	13.5
Xylose	36.5	63	<1	<1
Fructose	4	75	0	21

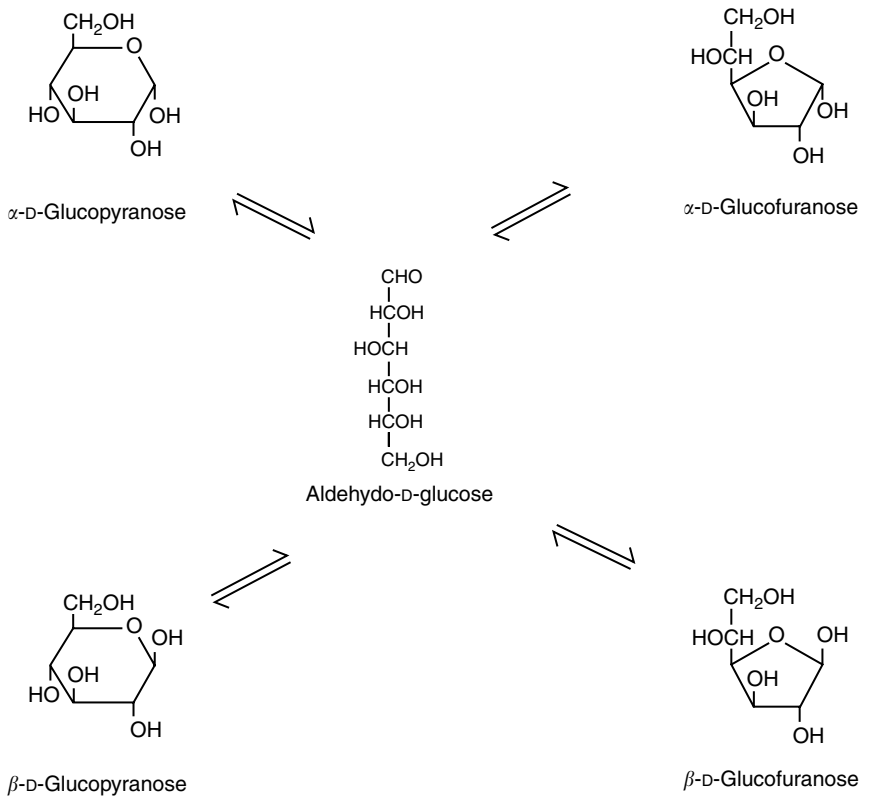

FIGURE 3.12 Interconversion of the acyclic and cyclic forms of D-glucose.

less methyl β-D-glucopyranoside (Figure 3.13). The two anomeric forms of the five-membered-ring furanosides are also formed; but being higher energy structures, they reorganize into more stable forms under the conditions of formation and are present at equilibrium in low amounts. The methyl group in this case, and any other group bonded to a sugar to make a glycoside, is termed an aglycon. Glycosides undergo hydrolysis in acidic environments to yield a reducing sugar (see Section 3.1.4.1) and a hydroxylated compound. Hydrolysis becomes more and more rapid as the temperature is raised.

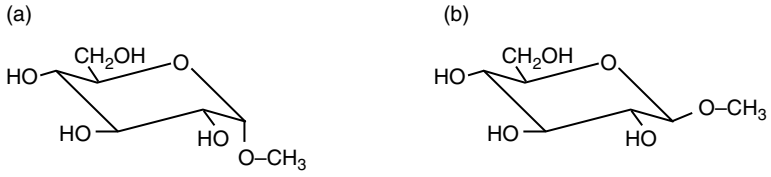

FIGURE 3.13 Methyl α-D-glucopyranoside (a) and methyl β-D-glucopyranoside (b).

3.1.4 Monosaccharide Reactions

All carbohydrate molecules have hydroxyl groups available for reaction. Simple monosaccharides and most other low-molecular-weight carbohydrate molecules also have carbonyl groups available for reaction. Formation of pyranose and furanose rings (cyclic hemiacetals) and glycosides (acetals) of monosaccharides have already been presented.

3.1.4.1 Oxidation to Aldonic Acids and Aldonolactones

Aldoses are readily oxidized to aldonic acids by oxidation of the aldehydo group to a carboxyl/carboxylate group. The reaction is commonly used for quantitative determination of sugars. One of the earliest methods for detection and measurement of sugars employed Fehling solution. Fehling solution is an alkaline solution of copper(II) that oxidizes an aldose to an aldonate and in the process is reduced to copper(I), which precipitates as brick-red Cu_2O. Variations (the Nelson–Somogyi and Benedict reagents) are still used for determining amounts of reducing sugars in foods and other biological materials.

$$2Cu(OH)_2 + R-\underset{\displaystyle H}{\underset{|}{C}}=O \longrightarrow R-\underset{\displaystyle O}{\underset{\|}{C}}-OH + Cu_2O + H_2O \qquad (3.1)$$

In the process of oxidizing the aldehydo group of an aldose to the salt of a carboxylic acid group, the oxidizing agent is reduced, that is, the sugar reduces the oxidizing agent; thus, aldoses are called reducing sugars. Ketoses are also termed reducing sugars because, under the alkaline conditions of the Fehling test, ketoses are isomerized to aldoses. The Benedict reagent, which is not alkaline, will react with aldoses, but not with ketoses.

A simple and specific method for quantitative oxidation of D-glucose to D-gluconic acid uses the enzyme glucose oxidase, the initial product being the 1,5-lactone (an intramolecular ester) of the acid (Figure 3.14). The reaction is commonly employed to measure the amount of D-glucose in foods and other biological materials, including the D-glucose concentration in blood and urine. D-Gluconic acid is a natural constituent of fruit juices and honey.

The reaction given in Figure 3.14 is also used for the manufacture of commercial D-gluconic acid and its lactone. D-Glucono-delta-lactone (GDL), D-glucono-1,5-lactone, according to systematic nomenclature, hydrolyzes largely to completion in water in about 3 h at room temperature, affecting a decrease in pH. Its slow hydrolysis, producing slow acidification and mild taste makes GDL unique among food acidulants. It is used in meats and dairy products, but particularly in refrigerated dough as a chemical leavening component.

3.1.4.2 Reduction of Carbonyl Groups

Hydrogenation is the addition of hydrogen to a double bond. When applied to carbohydrates, it entails addition of hydrogen to the double bond between the oxygen atom and the carbon atom of the carbonyl group of an aldose or ketose. Hydrogenation of D-glucose is readily accomplished with hydrogen gas under pressure in the presence of Raney nickel as a catalyst (Figure 3.15). The product

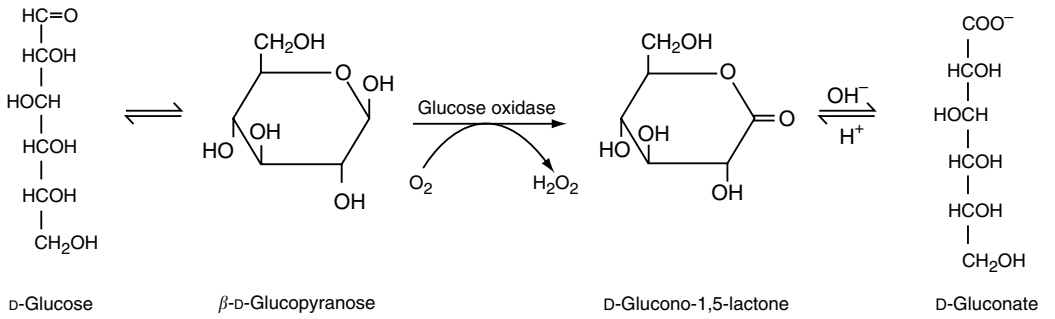

FIGURE 3.14 Oxidation of D-glucose catalyzed by glucose oxidase.

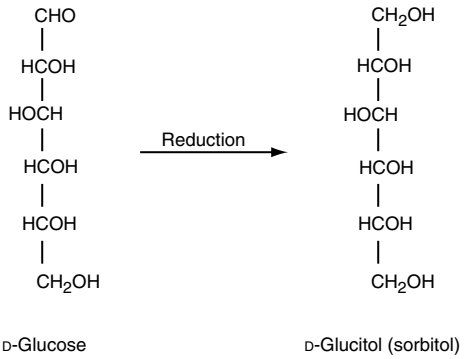

FIGURE 3.15 Reduction of D-glucose.

is D-glucitol, commonly known as sorbitol, the -itol suffix denoting a sugar alcohol (an alditol). Alditols are also known as polyols and polyhydroxy alcohols. Because it is derived from a hexose, D-glucitol (sorbitol) is specifically a hexitol. Sorbitol is widely distributed in plants, ranging from algae to higher plants, where it is found in fruits and berries; but the amounts present are generally small. It is about half as sweet as sucrose, is sold both as a syrup and as crystals, and is used as a general humectant, that is, a substance that will hold/retain moisture in a product.

D-Mannitol can be obtained by hydrogenation of D-mannose. Commercially, it is obtained along with sorbitol from hydrogenolysis of sucrose. It is a product of hydrogenation of the D-fructose (Figure 3.16) component of sucrose and from isomerization of D-glucose, which can be controlled by the alkalinity of the solution undergoing catalytic hydrogenation. D-Mannitol, unlike sorbitol, is not a humectant. Rather, it crystallizes easily and is only moderately soluble. It has been used as a nonsticky coating on candies. It is 65% as sweet as sucrose and is used in sugar-free chocolates, pressed mints, cough drops, and hard and soft candies.

Xylitol (Figure 3.17) is produced from hydrogenation of D-xylose obtained from hemicelluloses, especially from birch trees. Its crystals have a high negative heat of solution. This endothermic heat of solution of crystalline xylitol produces a cooling effect in the mouth. This cooling effect makes xylitol desirable as an ingredient in mint candies and in sugarless chewing gum. Its sweetness is about equal to that of sucrose. Xylitol is noncariogenic because it is not metabolized by the microflora of the mouth that produce dental plaques.

3.1.4.3 Uronic Acids

The terminal carbon atom (at the opposite end of the carbon chain from the aldehyde group) of a monosaccharide unit of an oligo- or polysaccharide may occur in an oxidized (carboxylic acid) form.

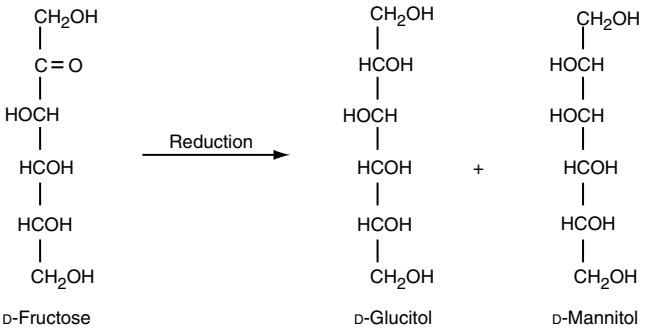

FIGURE 3.16 Reduction of D-fructose.

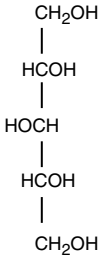

FIGURE 3.17 Xylitol.

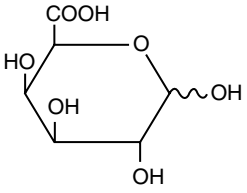

FIGURE 3.18 D-Galacturonic acid.

Such an aldohexose with C-6 in the form of a carboxylic acid group is called a uronic acid. When the chiral carbon atoms of a uronic acid are in the same configuration as they are in D-galactose, for example, the compound is D-galacturonic acid (Figure 3.18), the principal component of pectin (see Section 3.3.13).

3.1.4.4 Hydroxyl Group Esters

The hydroxyl groups of carbohydrates, like the hydroxyl groups of simple alcohols, form esters with organic and some inorganic acids. Reaction of hydroxyl groups with an activated form of a carboxylic acid, primarily a carboxylic acid anhydride, in the presence of a suitable base produces an ester:

$$\mathrm{ROH + R'-\overset{\overset{O}{\|}}{C}-O-\overset{\overset{O}{\|}}{C}-R' \quad or \quad R'-\overset{\overset{O}{\|}}{C}-Cl \rightarrow R-O-\overset{\overset{O}{\|}}{C}-R' + HO-\overset{\overset{O}{\|}}{C}-R' \quad or \quad HCl} \tag{3.2}$$

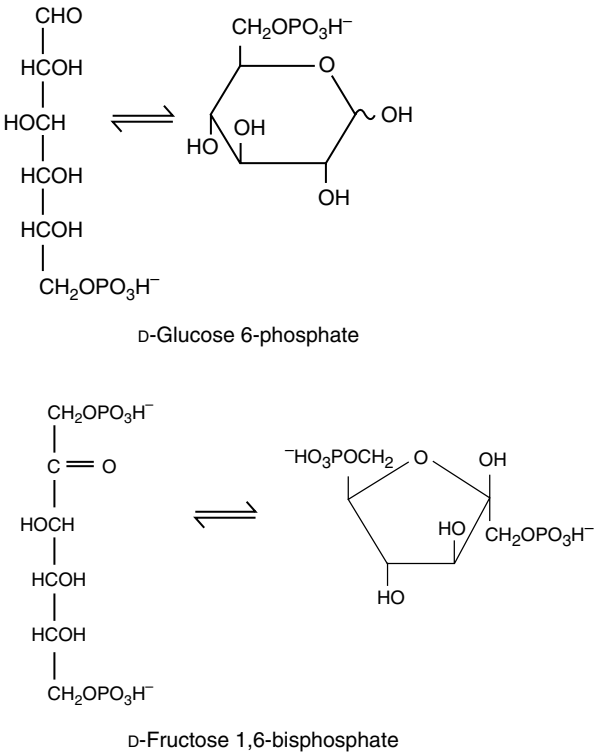

FIGURE 3.19 Examples of sugar phosphate metabolic intermediates.

Acetates, succinate half-esters, and other carboxylic acid esters of carbohydrates occur in nature. They are especially found as components of polysaccharides. Sugar phosphates are common metabolic intermediates (Figure 3.19).

Monoesters of phosphoric acid are also found as constituents of polysaccharides. For example, potato starch contains a small percentage of phosphate ester groups. Corn starch contains even less. In producing modified food starch, corn starch is often derivatized with mono- and distarch ester groups or both (see Section 3.3.6.10). Other esters of starch, most notably the acetate, succinate and substituted succinate half-esters, and distarch adipates, are modified food starches (see Section 3.3.6.10). Sucrose (see Section 3.2.3) fatty acid esters are produced commercially as water-in-oil emulsifiers. The family of red seaweed polysaccharides, which includes the carrageenans (see Section 3.3.10), contain sulfate groups (half-esters of sulfuric acid, $R{-}OSO_3^-$).

3.1.4.5 Hydroxyl Group Ethers

The hydroxyl groups of carbohydrates, like the hydroxyl groups of simple alcohols, can form ethers as well as esters. Ethers of carbohydrates are not as common in nature as are esters. However, polysaccharides are etherified commercially to modify their properties and make them more useful. Examples are the production of methyl ($-O-CH_3$), sodium carboxymethyl ($-O-CH_2-CO_2^-Na^+$), and hydroxypropyl ($-O-CH_2-CHOH-CH_3$) ethers of cellulose and hydroxypropyl ethers of starch, all of which are approved for food use.

A special type of ether, an internal ether linkage between carbon atoms 3 and 6 of a D-galactosyl unit (Figure 3.20), is found in the red seaweed polysaccharides, specifically agar, furcellaran, κ-carrageenan, and ι-carrageenan (see Section 3.3.10). Such an internal ether is known as a 3,6-anhydro ring; the name derives from the fact that it can be viewed as the product formed by removal of the elements of water (HOH) from the hydroxyl groups on C-3 and C-6.

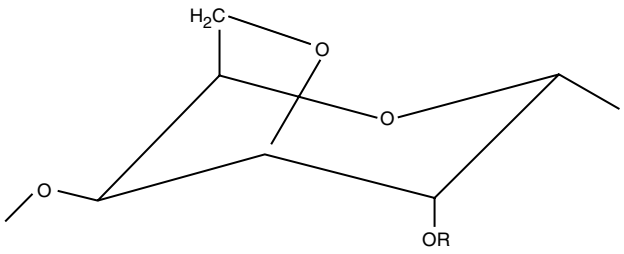

FIGURE 3.20 A 3,6-anhydro-α-D-galactopyranosyl unit found in red seaweed polysaccharides.

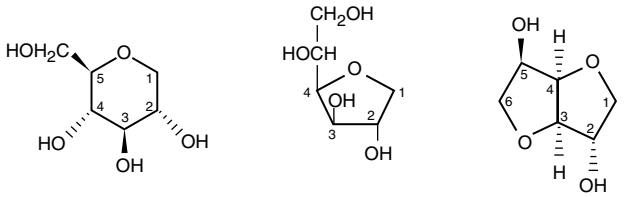

FIGURE 3.21 Anhydro-D-glucitols (sorbitans). Numbering refers to the carbon atoms in the original molecule of D-glucose (and of sorbitol).

A family of nonionic surfactants based on sorbitol (D-glucitol) are used in foods as water-in-oil emulsifiers and as defoamers. They are produced by esterification of sorbitol with fatty acids. Cyclic dehydration accompanies esterification (primarily at a primary hydroxyl group, that is, C-1 or C-6) so that the carbohydrate (hydrophilic) portion is, not only sorbitol, but also its mono- and dianhydrides (cyclic ethers of sorbitol called sorbitans, Figure 3.21). The products are known as sorbitan esters. Products called mono-, di-, and triesters (Spans) are formed. (The designation mono-, di-, and tri- simply indicates the ratio of fatty acid ester groups to sorbitan.) The product called sorbitan monostearate is actually a mixture of partial stearic (C_{18}) and palmitic (C_{16}) acid esters of sorbitol (D-glucitol), 1,5-anhydro-D-glucitol (1,5-sorbitan), 1,4-anhydro-D-glucitol (1,4-sorbitan), both internal (cyclic) ethers, and 1,4:3,6-dianhydro-D-glucitol (isosorbide), an internal dicyclic ether. Sorbitan fatty acid esters, such as sorbitan monostearate, sorbitan monolaurate, and sorbitan monooleate, are sometimes modified by reaction with ethylene oxide to produce so-called ethoxylated sorbitan esters called Tweens, which are also nonionic detergents approved by the U.S. FDA for food use.

3.1.4.6 Nonenzymic Browning [4,36,69]

Under some conditions, reducing sugars produce brown colors that are desirable and important in some foods. At other times, brown colors obtained upon heating or during long-term storage of foods containing reducing sugars are undesirable. Common browning of foods on heating or on storage is usually due to a chemical reaction between reducing sugars, mainly D-glucose, and a primary amino group (a free amino acid or amino group on a side chain of a protein molecule.) This reaction is called the Maillard reaction and the overall process is sometimes designated Maillard browning. It is also called nonenzymic or nonenzymatic browning to differentiate it from the more rapid, enzyme-catalyzed browning commonly observed in freshly cut fruits and vegetables, such as apples and potatoes.

When aldoses or ketoses are heated with amines, a variety of reactions ensue, producing numerous compounds (some of which are flavors, aromas, and dark-colored polymeric materials); but both reactants disappear only slowly. The flavors, aromas, and colors may be either desirable or undesirable. They may be produced slowly during storage and much more rapidly at the high temperatures encountered during frying, roasting, or baking.

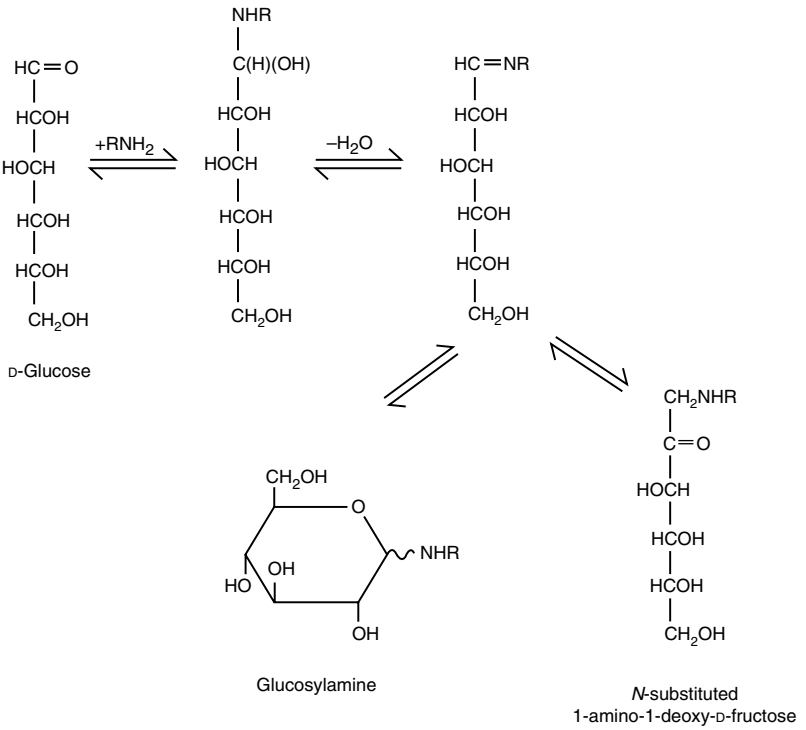

FIGURE 3.22 Products of reaction of D-glucose with a primary amine (RNH_2).

The reducing sugar reacts reversibly with the amine to form a Schiff base (an imine, RHC = NHR′), which may cyclize (in the same way that an aldose cyclizes) to form a glycosylamine (sometimes called a *N*-glycoside), as illustrated with D-glucose (Figure 3.22). The Schiff base undergoes a reaction called the Amadori rearrangement to give, in the case of D-glucose, a derivative of 1-amino-1-deoxy-D-fructose a so-called Amadori compound. Amadori compounds are early intermediates in the browning reaction sequence.

Amadori compounds undergo transformation via four known pathways starting with four different intermediates formed from them. The result is a complex mixture of intermediates and products. Three of the four intermediates formed by rearrangements and eliminations are 1-, 3-, and 4-deoxydicarbonyl compounds, usually known by their common names that are 1-, 3-, and 4-deoxyosones. Formation of these intermediates occurs most readily at pH 4–7. The most prevalent of these intermediates is usually the 3-deoxyosone (more properly called a 3-deoxyhexosulose, Figure 3.23).

Osones can cyclize in the same way that aldoses and ketoses do. They also will undergo dehydration, especially at high temperature. Reaction continues, especially at pH 5 or lower, to give an intermediate that dehydrates. Eventually, a furan derivative is formed: that from a hexose being 5-hydroxymethyl-2-furaldehyde, commonly known as hydroxymethylfurfural (HMF) (Figure 3.23); that formed from a pentose is furfural (furaldehyde). Under less acidic conditions, that is, pH > 5, the reactive cyclic compounds (HMF, furfural, and others) and compounds containing amino groups polymerize to a dark-colored, insoluble material containing nitrogen called melanoidin. Amino acids and furans (furfural and/or HMF) are almost always incorporated into the polymeric end products. Individual polymers constituting melanoidin vary in color (brown to black), molecular weight, nitrogen content, and solubility.

When higher concentrations of compounds containing primary amino groups (such as proteins containing higher proportions of L-lysine) are present, the primary products are pyrroles (products in which the ring oxygen atom of HMF and furfural is replaced with N—R).

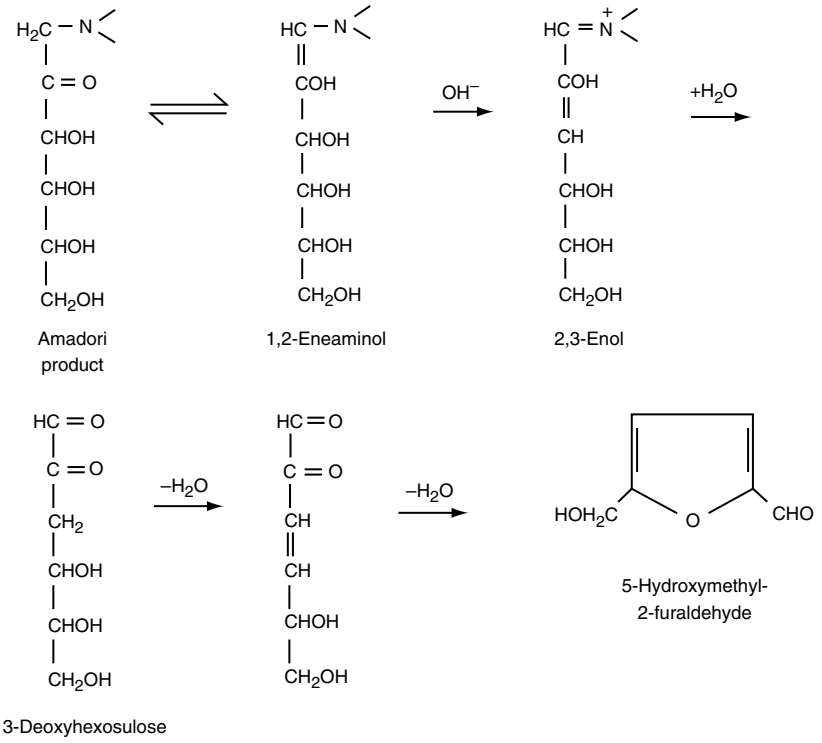

FIGURE 3.23 Conversion of the Amadori product into HMF.

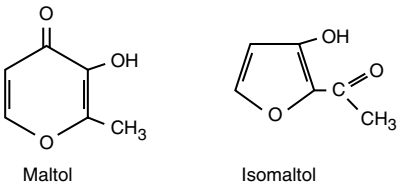

FIGURE 3.24 Maltol and isomaltol.

Maltol and isomaltol, both of which contribute to the flavor and aroma of bread, are formed from 1-deoxyosone (Figure 3.24).

Intermediates in the formation of melanoidin called reductones are also formed from 1-deoxyosones. Reductones are antioxidants. Because reductones can be involved in redox reactions, other intermediates can be formed from them (Figure 3.25).

Osones will also undergo cleavage, either between the two carbonyl groups or at the site of an enediol (—COH═COH—) forming shorter-chain products, primarily aldehydes that can undergo various reactions. Another important reaction of dicarbonyl compounds (osones and deoxyosones) is the Strecker degradation. Reaction of one of these compounds with an α-amino acid ($R—CHNH_2—CO_2H$) results first in a Schiff base being formed, then decarboxylation (releasing CO_2), dehydration, and elimination to produce an aldehyde that is one carbon atom shorter than the original amino acid. Aldehydes produced from amino acids often are major contributors to the aroma produced during nonenzymic browning. Among important aroma compounds produced in this way are 3-methylthiopropanal (methional, $CH_3—S—CH_2—CH_2—CHO$) from L-methionine, phenylacetaldehyde ($Ph—CH_2—CHO$) from L-phenylalanine, methylpropanal ($(CH_3)_2—CH—CHO$)

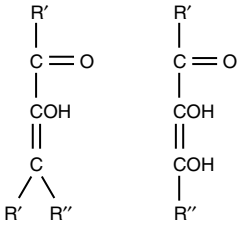

FIGURE 3.25 Two of several types of structures of reductones.

from L-valine, 3-methylbutanal ($(CH_3)_2{-}CH{-}CH_2{-}CHO$) from L-leucine, and 2-methylbutanol ($(CH_3{-}CH_2)\ (CH_3){-}CH{-}CHO$) from L-isoleucine.

A variety of the colored compounds collectively called melanoidins are formed. The variety arises from the variety of intermediates and the variety of possible condensation reactions. Some contain nitrogen; some contain only carbon, hydrogen, and oxygen atoms. All contain aromatic rings and conjugated double bonds.

Other products of the Maillard browning reaction are modified proteins. Protein modification primarily is the result of their reaction (especially reaction of the side chains of their L-lysine and L-arginine units) with carbonyl-group-containing compounds such as reducing sugars, osones, furfural, HMF, and pyrrole derivatives. For example, reaction of the ε-amino group of a unit of L-lysine in a protein molecule followed by the Amadori rearrangement converts the L-lysine unit into a unit of *N*-fructofuranosyl-lysine. Further reactions result in substituted furan and pyrrole rings being formed from the fructofuranosyl unit and being attached to the protein molecule. Reactions of this kind destroy the amino acid. Since L-lysine is an essential amino acid, its destruction in this way reduces the nutritional quality of the food. Losses of lysine and arginine of 15–40% in baked and roasted foods are common.

The product mixture formed is a function of temperature, time, pH, the nature of the reducing sugar, and the nature of the amino compound for the following reasons. Different sugars undergo nonenzymic browning at different rates. For example, D-glucose undergoes the browning reaction faster than does D-fructose. Secondary amines give different reaction products than do primary amines. Because the reaction has a relatively high energy of activation, application of heat is generally required. The rate of the Maillard reaction is also a function of the water activity (a_w) of a food product, reaching a maximum at a_w values in the range 0.6–0.7. Thus, for some foods, Maillard browning can be controlled by controlling water activity as well as by controlling reactant concentrations, time, temperature, and pH. Sulfur dioxide and bisulfite ions react with aldehyde groups, forming addition compounds, and thus will inhibit Maillard browning by removing at least some of a reactant (reducing sugar, HMF, furfural, etc.). Color, taste, and aroma are, in turn, determined by the product mixture. Reaction variables that can be controlled to increase or decrease the Maillard browning reaction are the following: (1) temperature (decreasing the temperature decreases the reaction rate) and time at the temperature; (2) pH (decreasing the pH decreases the reaction rate); (3) adjustment of the water content (maximum reaction rate occurs at water activity values of 0.6–0.7 [about 30% moisture]); (4) the specific sugar; and (5) presence of transition metal ions that undergo a one-electron oxidation under energetically favorable conditions, such as Fe(II) and Cu(I) ions (a free radical reaction may be involved near the end of the pigment-forming process).

In summary, Maillard browning products, including soluble and insoluble polymers, are found where reducing sugars and amino acids, proteins, and/or other nitrogen-containing compounds are heated together, for example, in soy sauce and bread crusts. Browning is desired in baking, for example, in bread crusts and cookies, and roasting of meats. The volatile compounds produced by nonenzymic browning (the Maillard reaction) during baking, frying, or roasting often provide desirable aromas. Maillard reaction products are also important contributors to the flavor of milk

chocolate, caramels, toffees, and fudges, during which reducing sugars react with milk proteins. The Maillard reaction also produces flavors, especially bitter substances, which may be desired, for example, in coffee. On the other hand, the Maillard reaction can result in off-flavors and off-aromas. Off-flavors and -aromas are most likely to be produced during pasteurization, storage of dehydrated foods, and grilling of meat or fish. Application of heat to intermediate moisture foods is generally required for nonenzymic browning.

3.1.4.7 Caramelization [4,59]

Heating of carbohydrates, in particular sucrose (Section 3.2.3) and reducing sugars, without nitrogen-containing compounds affects a complex group of reactions involved in caramelization. Reaction is facilitated by small amounts of acids and certain salts. Although it does not involve amino acids or proteins, carmelization is similar to nonenzymic browning. The final product, caramel (as in Maillard browning) contains a complex mixture of polymeric compounds, formed from unsaturated, cyclic (five- and six-membered ring) compounds. Also, as in Maillard browning, flavor and aroma compounds are also found. Heating causes dehydration of the sugar molecule with introduction of double bonds or formation of anhydro rings. As in Maillard browning, intermediates such as 3-deoxyosones and furans are formed. The unsaturated rings may condense to form useful, conjugated double-bond-containing, brown-colored polymers. Catalysts increase the reaction rate and are used to direct the reaction to specific types of caramel colors, solubilities, and acidities.

Caramel is produced commercially both as a coloring material and as a flavoring material. To make caramel, a carbohydrate is heated alone or in the presence of an acid, a base, or a salt. The carbohydrate most often used is sucrose, but D-fructose, D-glucose (dextrose), invert sugar, glucose syrups, HFSs, malt syrups, and molasses may also be used. Acids that may be used are food-grade sulfuric, sulfurous, phosphoric, acetic, and citric acids. Bases that may be used are ammonium, sodium, potassium, and calcium hydroxides. Salts that may be used are ammonium, sodium, and potassium carbonates, bicarbonates, phosphates (both mono- and dibasic), sulfates, and bisulfites. So, there are a very large number of variables, including temperature, in caramel manufacture. Ammonia may react with intermediates, such as 3-deoxyosones, produced by thermolysis to produce pyrazine and imidazole dervatives (Figure 3.26).

There are four recognized classes of caramel. Class I caramel (also called plain caramel or caustic caramel) is prepared by heating a carbohydrate without a source of either ammonium or sulfite ions; an acid or a base may be employed. Class II caramel (also called caustic sulfite caramel) is prepared by heating a carbohydrate in the presence of a sulfite, but in the absence of any ammonium ions; an acid or a base may be employed. This caramel, which is used to add color to beers and other alcoholic beverages, is reddish brown, contains colloidal particles with slightly negative charges, and has a solution pH of 3–4. Class III caramel (also called ammonium caramel) is prepared by heating a carbohydrate in the presence of a source of ammonium ions, but in the absence of sulfite ions; an acid or a base may be employed. This caramel, which is used in bakery products, syrups, and puddings, is reddish brown, contains colloidal particles with positive charges, and gives a solution

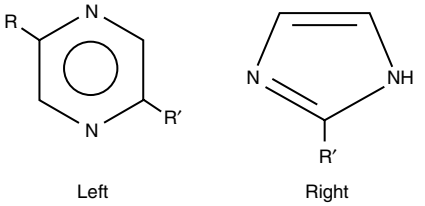

FIGURE 3.26 Pyrazine (left) and imadazole (right) derivatives formed during carmelization in the presence of ammonia R = $-CH_2-(CHOH)_2-CH_2OH$, R′ = $-(CHOH)_3-CH_2OH$.

pH of 4.2–4.8. Class IV caramel (also called sulfite ammonium caramel) is prepared by heating a carbohydrate in the presence of both sulfite and ammonium ions; an acid or a base may be employed. This caramel, which is used in cola soft drinks, other acidic beverages, baked goods, syrups, candies, pet foods, and dry seasonings, is brown, contains colloidal particles with negative charges, and gives a solution pH of 2–4.5. In this case, the acidic salt catalyzes cleavage of the glycosidic bond of sucrose, and the ammonium ion participates in the Amadori rearrangement reaction. The pigments in all four types of caramel are large polymeric molecules with complex, variable, and unknown structures. It is these polymers that form the colloidal particles. Their rate of formation increases with increasing temperature and pH. Of course, caramelization may also occur during cooking or baking, especially when sugar is present. It occurs along with nonenzymic browning during the preparation of chocolate and fudge.

3.1.4.8 Formation of Acrylamide in Food [3,18,50,70,73]

The Maillard reaction has been implicated in the formation of acrylamide in many foods that have been heated to high temperatures during processing or preparation. Levels of acrylamide (typically <1.5 ppm) have been reported in a wide range of food products that are made by frying, baking, puffing, roasting, or other elevated-temperature processing schemes during production or preparation (Table 3.3). Acrylamide is not detected in unheated or even boiled foodstuffs, such as boiled potatoes, because the temperature during boiling does not go above $\sim$100°C. Acrylamide is undetected or detected at only very low levels in canned or frozen fruits, vegetables, and vegetable protein products (vegetable burgers and related products) with the exception of pitted ripe olives, in which the measured levels ranged from 0 to 1925 ppb. Acrylamide is a known neurotoxicant and probably a weak human carcinogen at exposure levels much higher than are obtained from food.

Acrylamide is derived primarily from the second-order reaction between reducing sugars (carbonyl moiety) and the α-amino group of free L-asparagine (Figure 3.27). The reaction requires

TABLE 3.3
Ranges of Acrylamide Found in Common Food Products Containing High Levels

Food	ppb Acrylamide[a]
Almonds (roasted)	236–457
Bagels	0–343
Breads	0–364
Breakfast cereals (RTE)	34–1057
Cocoa	0–909
Coffee (unbrewed)	3–374
Coffee with chicory	380–609
Cookies	36–432
Crackers and related products	26–1540
French fries	20–1325
Potato chips	117–196[b]
Pretzels	46–386
Tortillas	10–33
Tortilla chips	117–196

[a] Extreme values, especially extremely high values, are usually representative of only a small number of sampled products.
[b] A sample of sweet potato chips contained 4080 ppb acrylamide.

Source: Center for Food Safety and Applied Nutrition, USDA.

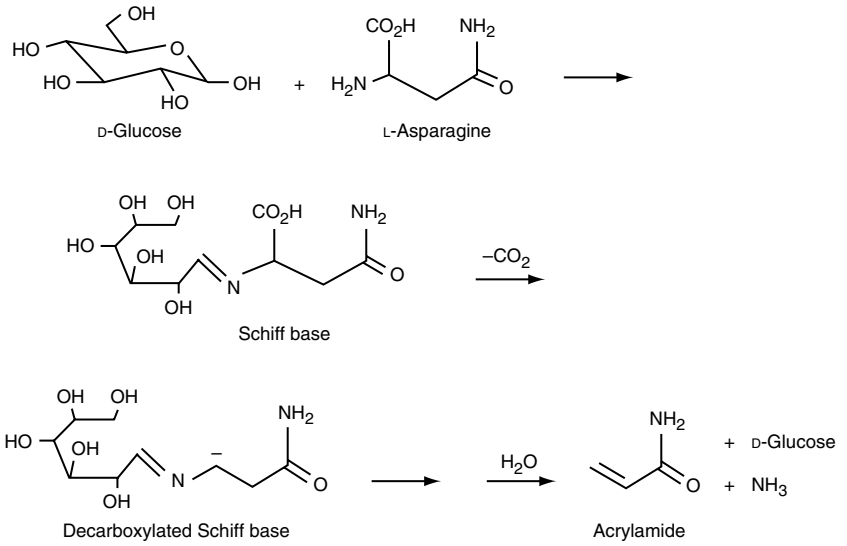

FIGURE 3.27 A proposed mechanism of acrylamide formation in foods.

the presence of both substrates. Fried potato products, such as potato chips and French fries, are particularly susceptible to acrylamide formation because potatoes contain both free D-glucose and free L-aspargine. The reaction most likely occurs via a Schiff base intermediate, which then undergoes decarboxylation, followed by carbon–carbon bond cleavage to form acrylamide, whose atoms are known to be derived solely from L-asparagine. Though acrylamide is not the favored product of this complex series of reactions (reaction efficiency $\approx 0.1\%$), it is able to accumulate to detectable levels in food products subjected to prolonged heating at high temperatures. Acrylamide formation requires a minimum temperature of 120°C, which means that it cannot occur in high-moisture foods, and is kinetically favored with increasing temperatures approaching 200°C. With extended heating at temperatures above 200°C, acrylamide levels may actually decrease via thermal elimination/degradation reactions. Food levels of acrylamide are also impacted by pH. Acrylamide formation is favored as the pH is increased over the range of 4–8. Reduced acrylamide formation in the acid range is thought to be due in part to protonation of the α-amino group of asparagine, reducing its nucleophilic potential. Furthermore, acrylamide appears to undergo increased rates of thermal degradation as the pH decreases. Acrylamide levels increase rapidly in the latter stages of the prolonged heating process as the water at food surfaces is driven off to allow surface temperatures to increase above 120°C. Products with high amounts of surface area, such as potato chips, are among those high-temperature processed foods that exhibit the highest acrylamide levels. Thus, exposed surface area of a food can be an additional factor, provided that reaction substrates and processing temperatures are sufficient for acrylamide formation.

Efforts to minimize formation of acrylamide in food generally involve one or more of three strategies: (1) removal of either one or both of the substrates, (2) alteration of processing conditions, and (3) acrylamide removal from food following its formation. Through blanching or soaking in water, it is possible to achieve up to a 60% reduction in acrylamide levels within processed potato products through removal of reaction substrates (reducing sugars and free asparagine). Reagent modification (e.g., protonation of asparagine by lowering the pH or conversion of asparagine to aspartic acid with asparaginase), addition of competing substrates that do not yield acrylamide (e.g., amino acids other than asparagine or protein), and incorporation of salts have been shown to mitigate acrylamide formation. Where possible, better control or optimization of thermal processing conditions (temperature/time relationships) may also prove beneficial to minimizing acrylamide

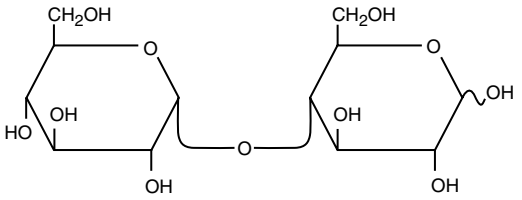

FIGURE 3.28 Maltose.

levels. It is likely that a combination of mitigation methods will be required to effectively limit acrylamide formation within food products, with the employed methods likely varying according to the nature and needs of a particular food system.

Although studies to date have uncovered no association between acrylamide consumption in foods and the risk of cancer, long-term carcinogenicity, mutagenicity, and neurotoxicity studies are still ongoing as are efforts to reduce acrylamide formation during food processing and preparation.

3.2 OLIGOSACCHARIDES

An oligosaccharide contains from 2 to 10 or from 2 to 20 sugar units, depending on who is defining the term, joined by glycosidic bonds. When a molecule contains more than 20 units, it is a polysaccharide.

Disaccharides are glycosides in which the aglycon is a monosaccharide unit. A compound containing three monosaccharide units is a trisaccharide. Structures containing from 4 to 10 glycosyl units, whether linear or branched, are tetra-, penta-, hexa-, octa-, nona-, and decasaccharides, and so on. Only a few oligosaccharides occur in nature. Most are produced by hydrolysis of polysaccharides into smaller units. Because glycosidic bonds are part of acetal structures, they undergo acid-catalyzed hydrolysis in the presence of aqueous acid and heat.

3.2.1 Maltose

Maltose (Figure 3.28) is an example of a disaccharide. The reducing end unit (on the right as customarily written) has a potentially free aldehyde group and in solution will be in equilibrium with alpha and beta six-membered ring forms, as described earlier for monosaccharides. Since O-4 is blocked by attachment of the second D-glucopyranosyl unit, a furanose ring cannot form. Maltose is a reducing sugar, because its aldehyde group is free to react with oxidants and, in fact, to undergo almost all reactions as though it were present as a free aldose.

Maltose is produced by hydrolysis of starch using the enzyme β-amylase (see Section 3.3.6.9). It occurs only rarely in nature and only in plants as a result of partial hydrolysis of starch. Maltose is produced during malting of grains, especially barley, and commercially by the specific enzyme-catalyzed hydrolysis of starch using β-amylase from *Bacillus* species, although the β-amylases from barley seed, soybeans, and sweet potatoes may be used. Maltose is used sparingly as a mild sweetener for foods. Maltose is reduced to the alditol maltitol, which is used in sugarless chocolate.

3.2.2 Lactose

The disaccharide lactose (Figure 3.29) occurs in milk, mainly free, but to a small extent as a component of higher oligosaccharides. The concentration of lactose in milk varies with the mammalian source from 2.0 to 8.5%. Cow and goat milk contains 4.5–4.8%, human milk about 7%. Lactose is the primary carbohydrate source for developing mammals. In humans, lactose constitutes 40% of the energy consumed during nursing. Utilization of lactose for energy must be preceded by hydrolysis to the constituent monosaccharides, D-glucose and D-galactose, because only monosaccharides are

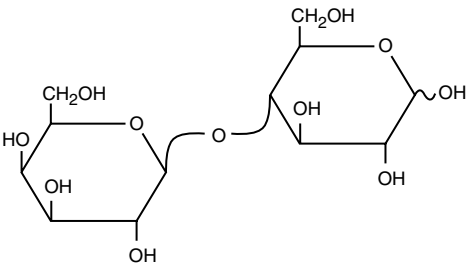

FIGURE 3.29 Lactose.

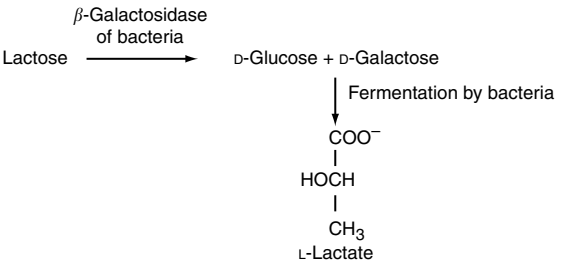

FIGURE 3.30 The fate of lactose in the large intestine of persons with lactase deficiency.

absorbed from the small intestine. Milk also contains 0.3–0.6% of lactose-containing oligosaccharides, many of which are important as energy sources for growth of a specific variant of *Lactobacillus bifidus*, which, as a result, is the predominant microorganism of the intestinal flora of breast-fed infants.

Lactose is ingested in milk and other unfermented dairy products, such as ice cream. Fermented dairy products, such as most yogurt and cheese, contain less lactose because, during fermentation, some of the lactose is converted into lactic acid. Lactose stimulates intestinal adsorption and retention of calcium. Lactose is not digested until it reaches the small intestine, where the hydrolytic enzyme lactase is present. Lactase (a β-galactosidase) is a membrane-bound enzyme located in the brush border epithelial cells of the small intestine. It catalyzes the hydrolysis of lactose into its constituent monosaccharides, D-glucose and D-galactose, both of which are rapidly absorbed and enter the blood stream:

$$\text{lactose} \xrightarrow{\text{lactase}} \text{D-glucose} + \text{D-galactose} \tag{3.3}$$

If for some reason the ingested lactose is only partially hydrolyzed, that is, only partially digested, or is not hydrolyzed at all, a clinical syndrome called lactose intolerance results. If there is a deficiency of lactase, some lactose remains in the lumen of the small intestine. The presence of lactose tends to draw fluid into the lumen by osmosis. This fluid produces abdominal distention and cramps. From the small intestine, the lactose passes into the large intestine (colon) where it undergoes anaerobic bacterial fermentation to lactic acid (present as the lactate anion) (Figure 3.30) and other short-chain acids. The increase in the concentration of molecules, that is, the increase in osmotic strength, results in still greater retention of fluid. In addition, the acidic products of fermentation lower the pH and irritate the lining of the colon, leading to an increased movement of the contents. Diarrhea is caused both by the retention of fluid and the increased movement of the intestinal contents. The gaseous products of fermentation cause bloating and cramping.

Lactose intolerance is not usually seen in children until after about 6 years of age. At this point, the incidence of lactose-intolerant individuals begins to rise and increases throughout the

life span with the greatest incidence in the elderly. Both the incidence and the degrees of lactose intolerance vary by ethnic group, indicating that the presence or absence of lactase is under genetic control.

There are three ways to overcome the effects of lactase deficiency. One is to remove the lactose by fermentation as in yogurt and cultured buttermilk products. Another is to produce reduced-lactose milk by adding lactase to it. However, both products of hydrolysis, D-glucose and D-galactose, are sweeter than lactose, and at about 80% hydrolysis, the taste change becomes quite evident. Therefore, most reduced-lactose milk has the lactose reduced as close as possible to the 70% government-mandated limit for a claim. The third is for the lactase-deficient individual to consume β-galactosidase along with the dairy product.

3.2.3 Sucrose [40,46]

When the total amount of sucrose, usually called simply sugar or table sugar, used in the United States is divided by the total population, it is calculated that the per person daily utilization averages about 160 g; but sucrose is also used extensively in fermentations, in bakery products where it is also largely used up in fermentation, and in pet food; so the actual average daily amount consumed by individuals in foods and beverages is much less, estimated to be about 55 g (20 kg or 43 lb/yr). Sucrose is composed of an α-D-glucopyranosyl unit and a β-D-fructofuranosyl unit linked head-to-head (reducing end-to-reducing end) rather than by the usual head-to-tail linkage (Figure 3.31). Since it has no reducing end, it is classified as a nonreducing sugar.

There are two principal sources of commercial sucrose—sugar cane and sugar beets. Also present in sugar beet extract are (1) a trisaccharide, raffinose, which has a D-galactopyranosyl unit attached to sucrose and (2) a tetrasaccharide, stachyose, which contains another D-galactosyl unit (Figure 3.32). These oligosaccharides, also found in beans, are nondigestible. These and other carbohydrates that are not completely broken down into monosaccharides by intestinal enzymes and are not absorbed pass into the colon. There, they are metabolized by microorganisms producing lactate and gas. Diarrhea, bloating, and flatulence result.

Sucrose has a specific optical rotation of $+66.5°$. The equimolar mixture of D-glucose and D-fructose produced by hydrolysis of the glycosidic bond joining the two monosaccharide units has a specific optical rotation of $-33.3°$. Early investigators, noticing this, called the process inversion and the product invert sugar.

Sucrose and most other low-molecular-weight carbohydrates (e.g., monosaccharides, alditols, disaccharides, and other low-molecular-weight oligosaccharides), because of their great hydrophilicity and solubility, can form highly concentrated solutions of high osmolality. Such solutions, as

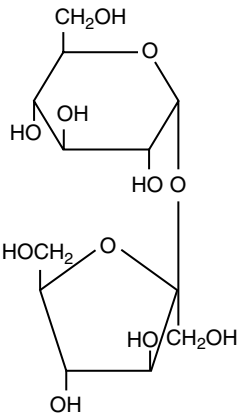

FIGURE 3.31 Sucrose.

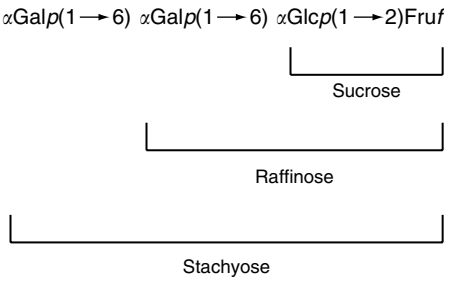

FIGURE 3.32 Sucrose, raffinose, and stachyose. (For explanation of the shorthand designations of structures, see Section 3.3.1.)

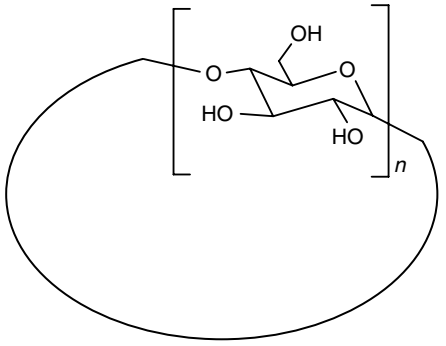

FIGURE 3.33 Generalized chemical structures of α- ($n = 6$), β- ($n = 7$), and γ- ($n = 8$) cyclodextrins.

exemplified by honey, need no preservatives themselves and can be used, not only as sweeteners (although not all such carbohydrate syrups need have much sweetness), but also as preservatives and humectants.

A portion of the water in any carbohydrate solution is nonfreezable. When the freezable water crystallizes, that is, forms ice, the concentration of solute in the remaining liquid phase increases, and the freezing point decreases. There is a consequential increase in viscosity of the remaining solution. Eventually, the liquid phase solidifies as a glass in which the mobility of all molecules becomes restricted and diffusion-dependent reactions become very slow (see Chapter 2) and, because of the restricted motion, water molecules become unfreezable, that is, they cannot form crystals. In this way, carbohydrates function as cryoprotectants and protect against the dehydration that destroys structure and texture caused by freezing.

The sucrase of the human intestinal tract catalyzes hydrolysis of sucrose into D-glucose and D-fructose, making sucrose one of the three carbohydrates humans can digest and utilize for energy, the other two being lactose and starch. Monosaccharides (D-glucose and D-fructose being the nutritionally significant ones in our diets) do not need to be transformed before absorption.

3.2.4 Cyclodextrins [48,56]

Cyclodextrins, formerly known as Schardinger dextrins and cycloamyloses, comprise a family of cyclic oligosaccharides comprised of (1→4)-linked α-D-glucopyranosyl units (Figure 3.33). These cyclic structures are formed from soluble, partially hydrolyzed starch polymers (Section 3.3.6.9) through action of the enzyme, cyclodextrin glycosyltransferase (CGTase), which catalyzes the intramolecular cyclization of glucosyl chains. Cyclodextrins consist of six, seven, or eight glucosyl

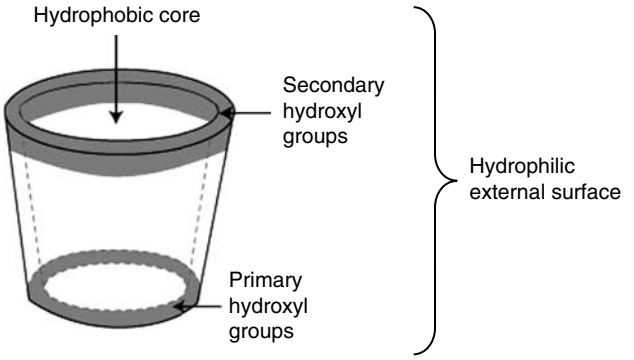

FIGURE 3.34 Depiction of the idealized geometric shape of cyclodextrins.

TABLE 3.4
Chemical Characteristics of α-, β-, and γ-Cyclodextrins

Characteristic	α	β	γ
No. of glucosyl units	6	7	8
Molecular weight	972	1135	1297
Solubility (g/100 mL at 25°C)	14.5	1.9	23.2
Cavity diameter (Å)	4.7–5.3	6.0–6.5	7.5–8.3

units; these cyclodextrins are referred to as α-, β-, and γ-cyclodextrins, respectively. In commercial production schemes, cyclodextrins may be isolated by selective crystallization (following treatment of the reaction broth with glucoamylase) or differential precipitation involving addition of a substrate-specific complexing agent (typically an organic solvent). While α-, β-, and γ-cyclodextrins are all permitted for use in food (self-affirmed GRAS regulatory status), only β-cyclodextrin is utilized to any appreciable degree due to its lower cost (relative to the other two) and established function.

Cyclodextrins possess a truncated funnel- or doughnutlike geometry with an internal hydrophobic core or cavity and a hydrophilic external surface (Figure 3.34). The solubility of cydodextrins in water, which is attributable to the presence of the hydroxyl groups on their outer molecular surface, is different for α-, β-, and γ-types (Table 3.4). γ-Cyclodextrin is the most water soluble, followed by α-cyclodextrin, while the β-type, due to an extensive band of intramolecular hydrogen bonds spanning the entire outer molecular perimeter, has the lowest water solubility. In contrast, the internal cavity provides a hydrophobic environment for formation of inclusion complexes with nonpolar guest molecules through hydrophobic and other noncovalent associations. The size of the inner cavity increases as the number of cyclodextrin glycosyl units increases ($\gamma > \beta > \alpha$) (Table 3.4). This complexing ability is the most significant property of cyclodextrins and is the driving force for cyclodextrin use in almost all food and industrial applications. Within food systems, cyclodextrins may be used to complex flavors, lipids, and color compounds for an array of purposes. Cyclodextrins may be used to complex undesirable constituents (such as masking of off-flavors, odors, and bitter compounds and removal of cholesterol and free fatty acids [FFAs]), to stabilize against chemical oxidation (e.g., protection of flavor compounds, binding of enzymic browning phenolic precursors), to enhance nonwater-soluble (lipophilic) flavor compounds, and to improve physical stability of food ingredients (encapsulation of volatiles, controlled release of flavor).

3.3 POLYSACCHARIDES [54,65]

3.3.1 Polysaccharide Chemical Structures and Properties

Polysaccharides are polymers of monosaccharides. Like the oligosaccharides, they are composed of glycosyl units in linear or branched arrangements, but most are much larger than the 10- or 20-unit limit of oligosaccharides. The number of monosaccharide units in a polysaccharide, which is termed its degree of polymerization (DP), varies. Only a few polysaccharides have DPs less than 100; most have DPs in the range 200–3,000. The larger ones, such as cellulose, have a DP of 7,000–15,000. Starch amylopectin is even larger, having an average molecular weight of at least 10^7 (DP > 60,000). It is estimated that more than 90% of the carbohydrate mass in nature is in the form of polysaccharides. The general scientific term for polysaccharides is glycans.

If all the glycosyl units are of the same sugar type, they are homogeneous as to monomer units and are called homoglycans. Examples of homoglycans are cellulose (Section 3.3.7) and starch amylose (Section 3.3.6.1), which are linear, and amylopectin (Section 3.3.6.2), which is branched. All three are composed only of D-glucopyranosyl units.

When a polysaccharide is composed of two or more different monosaccharide units, it is a heteroglycan. A polysaccharide that contains two different monosaccharide units is a diheteroglycan; a polysaccharide that contains three different monosaccharide units is a triheteroglycan, and so on. Diheteroglycans generally are either linear polymers of blocks of similar units alternating along the chain, or consist of a linear chain of one type of glycosyl unit with a second present as single-unit branches. Examples of the former type are algins (Section 3.3.11) and of the latter guar and locust bean gums (LBGs) (Section 3.3.8).

In the shorthand notations of oligo- and polysaccharides, the glycosyl units are designated by the first three letters of their names with the first letter being capitalized, except for glucose which is Glc. If the monosaccharide unit is that of a D-sugar, the D is omitted; only L-sugars are so designated, for example, L-Ara for L-arabinose. The size of the ring is designated by an italicized *p* for pyranose or *f* for furanose. The anomeric configuration is designated with α or β as appropriate, for example, an α-D-glucopyranosyl unit is indicated as αGlc*p*. Uronic acids are designated with a capital A, for example, an L-gulopyranosyluronic acid unit (see Section 3.3.11) is indicated as LGul*p*A. The position of linkages are designated either as, for example, 1→3 or 1,3, the latter being more commonly used by biochemists and the former more commonly used by carbohydrate chemists. Using the shorthand notation, the structure of lactose is represented as βGal*p*(1→4)Glc or βGal*p*1,4Glc and maltose as αGlc*p*(1→4)Glc or αGlc*p*1,4Glc. Note that the reducing end cannot be designated as α or β or as being in a pyranose or furanose ring (except in the case of a crystalline product) because the ring can open and close; that is, in solutions of both lactose and maltose and other oligo- and polysaccharides, the reducing end unit will occur as a mixture of α- and β-pyranose ring forms and the acyclic form, with rapid interconversion between them (see Figure 3.12).

3.3.2 Polysaccharide Solubility

Most polysaccharides contain glycosyl units that, on average, have three hydroxyl groups. Each of the hydroxyl groups has the possibility of hydrogen bonding to one or more water molecules. Also, the ring oxygen atom and the glycosidic oxygen atom connecting one sugar ring to another can form hydrogen bonds with water. With every sugar unit in the chain having the capacity to hold water molecules, glycans possess a strong affinity for water and most hydrate readily when water is available. In aqueous systems, polysaccharide particles can take up water, swell, and usually undergo partial or complete dissolution.

Polysaccharides, like lower-molecular-weight carbohydrates, modify and control the mobility of water in food systems, and water plays an important role in influencing the physical and functional properties of polysaccharides. Polysaccharides and water together control many functional properties of foods, including texture.

The water of hydration that is naturally hydrogen bonded to polysaccharide molecules is often described as nonfreezable water, that is, water whose structure has been sufficiently modified by the presence of the polymer molecule so that it will not freeze. This water has also been referred to as plasticizing water. The molecules that make up this water are not energetically bound in a chemical sense. While their motions are retarded, they are able to exchange freely and rapidly with other water molecules. This water of hydration makes up only a small part of the total water in gels and fresh tissue foods. Water in excess of the hydration water is entrapped in capillaries and cavities of various sizes in the gel or tissue.

Polysaccharides are cryostabilizers, rather than cryoprotectants. They do not increase the osmolality or depress the freezing point of water significantly, because they are large, high-molecular-weight molecules and osmotic strength and freezing point depression are colligative properties. When a polysaccharide solution is frozen, a two-phase system of crystalline water (ice) and a glass consisting of perhaps 70% polysaccharide molecules and 30% nonfreezable water is formed. As in the case of solutions of low-molecular-weight carbohydrates, the nonfreezable water is part of a highly concentrated solution in which the mobility of the water molecules is restricted by the extremely high viscosity. While some polysaccharides provide cryostabilization by producing this freeze-concentrated matrix that severely limits molecular mobility, others provide cryostabilization by restricting ice crystal growth by adsorption to nuclei or active crystal growth sites. Some polysaccharides in nature are ice nucleators.

So both high- and low-molecular-weight carbohydrates are generally effective in protecting food products stored at freezer temperatures (typically −18°C) from destructive changes in texture and structure, with various degrees of effectiveness. The improvement in product quality and storage stability is a result of controlling both the amount (particularly in the case of low-molecular-weight carbohydrates) and the structural state (particularly in the case of polymeric carbohydrates) of the freeze-concentrated, amorphous matrix surrounding ice crystals.

Most, if not all, polysaccharides, except those with very bushlike, branch-on-branch structures, exist in some sort of helical shape. Certain linear homoglycans, such as cellulose (see Section 3.3.7), have flat, ribbon-like structures. Such uniform linear chains undergo hydrogen bonding with each other so as to form crystallites separated by amorphous regions (Figure 3.35). It is these crystallites of linear chains that give cellulose fibers, such as wood and cotton fibers, their great strength, insolubility, and resistance to breakdown; the latter because the crystalline regions are nearly inaccessible to enzyme penetration. These polysaccharides with high degrees of orientation and crystallinity are exceptions. Most polysaccharides are not so crystalline and readily hydrate and dissolve in water.

Unbranched diheteroglycans containing nonuniform blocks of glycosyl units and most branched polysaccharides cannot form micelles because their chain segments are prevented from becoming closely packed over lengths necessary to provide enough intermolecular bonding to form sizeable crystallites. Hence, these chains have a degree of solubility that increases as chains become less able to fit closely together. In general, polysaccharides become more soluble in proportion to the degree of irregularity of the molecular chains, which is another way of saying that, as the ease with which molecules fit together decreases, the solubility of the molecules increases.

Water-soluble polysaccharides and modified polysaccharides used in food and other industrial applications are known as gums or hydrocolloids. Food gums are sold as powders of varying particle size.

3.3.3 Polysaccharide Solution Viscosity and Stability [12,20]

Polysaccharides (gums, hydrocolloids) are used in foods primarily to thicken and/or gel aqueous systems and otherwise to modify and/or control the flow properties and textures of liquid products and the deformation properties of semisolid products. They are generally used in food products at concentrations of 0.25–0.50%, indicating their great ability to produce viscosity and to form gels.

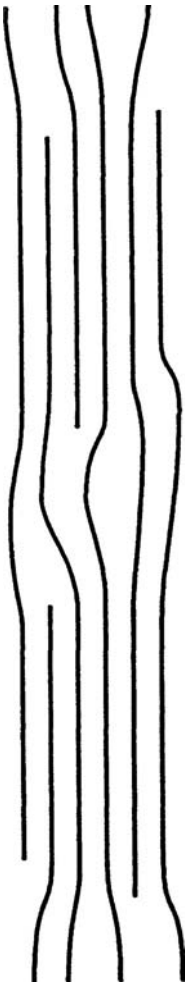

FIGURE 3.35 Crystalline regions in which the chains are parallel and ordered separated by amorphous regions.

The viscosity of a polymer solution is a function of the size and shape of its molecules and the conformations they adopt in the solvent. In foods and beverages, the solvent is an aqueous solution of other solutes. The shapes of polysaccharide molecules in solution are a function of rotations around the bonds of the glycosidic linkages. The greater the internal freedom at each glycosidic linkage, the greater the number of conformations available to each individual segment. Chain flexibility provides a strong entropic drive, which generally overcomes energy considerations and induces the chain to approach disordered or random coil (Figure 3.36) states in solution. However, most polysaccharides exhibit deviations from strictly random coil states, forming stiff coils, the specific nature of the coils being a function of the monosaccharide composition and linkages.

The motion of linear polymer molecules in solution results in their sweeping out a large space. When they collide with each other, they create friction, consume energy, and thereby produce viscosity. Linear polysaccharides produce highly viscous solutions, even at low concentrations. Viscosity depends both on the DP (molecular weight) and the shape and flexibility of the solvated polymer chain, with the longer, more extended, and/or more rigid molecules producing the greatest viscosity. With respect to DP, carboxymethylcellulose (CMC) (see Section 3.3.7.2) and products obtained from the parent CMC can have solution viscosities at 2% concentration that can vary from <5 to $>100{,}000$ mPa · S.

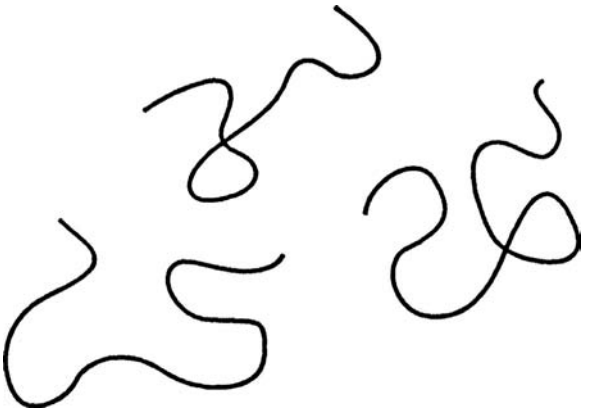

FIGURE 3.36 Randomly coiled polysaccharide molecules.

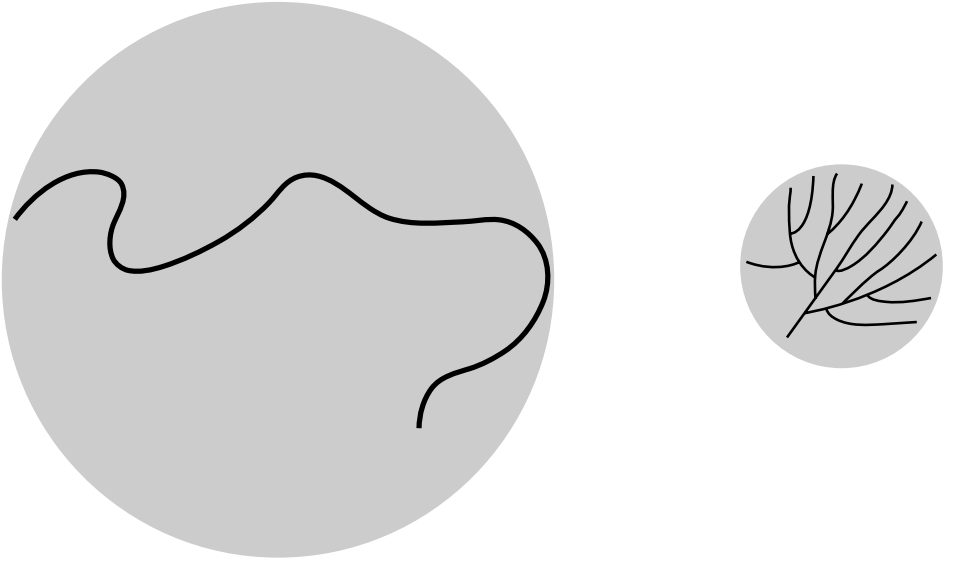

FIGURE 3.37 Relative volumes occupied by a linear polysaccharide and a highly branched polysaccharide of the same molecular weight.

A highly branched polysaccharide will sweep out much less space than a linear polysaccharide of the same molecular weight (Figure 3.37). As a result, highly branched molecules will collide less frequently and will produce a much lower viscosity than will linear molecules of the same DP. This also implies that a highly branched polysaccharide must be significantly larger than a linear polysaccharide to produce the same viscosity at the same concentration.

Likewise, linear polysaccharide chains bearing only one type of ionic charge (almost always a negative charge imparted by ionized carboxyl or sulfate half-ester groups) cause them to assume an extended configuration due to repulsion of the like charges, increasing the end-to-end chain length and, thus, increasing the volume swept out by the polymer. Therefore, these polymers tend to produce solutions of high viscosity.

Unbranched glycans with regular repeating unit structures form unstable aqueous dispersions that precipitate or gel rapidly. This occurs as segments of the long molecules collide and form intermolecular bonds over the distance of a few units. Initial short alignments then extend in a

zipper-like fashion to greatly strengthen intermolecular associations. Other segments of other chains colliding with this organized nucleus bind to it, increasing the size of the ordered, crystalline phase. Linear molecules continue to bind to fashion a fringed micelle that may reach a size where gravitational forces cause precipitation. For example, starch amylose, when dissolved in water with the aid of heat and then cooled to below 65°C, undergoes molecular aggregation and precipitates, a process called retrogradation. During cooling of bread and other baked products, amylose molecules associate to produce firming. Over a longer storage time, the branches of amylopectin associate (and may partially crystallize) to produce staling (Section 3.3.6.7).

In general, molecules of unbranched, neutral homoglycans have an inherent tendency to associate and partially crystallize. However, if linear glycans are derivatized, or occur naturally derivatized, as does guar gum (Section 3.3.8), which has single-unit glycosyl branches along a backbone chain, their segments are prevented from association and stable solutions result.

Stable solutions are also formed if the linear chains contain charged groups so that Coulombic repulsions prevent segments from approaching each other. As already mentioned, charge repulsion also causes chains to extend, which provides high viscosities. Such highly viscous, stable solutions are seen with sodium alginate (Section 3.3.11), where each glycosyl unit is a uronic acid unit having a carboxylic acid group in the salt form, and in xanthan (Section 3.3.9), where one out of five glycosyl units is a uronic acid unit and another carboxylate group from a cyclic acetal of pyruvic acid is present at a frequency of about one per every ten monosaccharide units. But, if the pH of an alginate solution is lowered to 3, where ionization of carboxylic acid groups is repressed because the pK_a values of the constituent monomers are 3.38 and 3.65, the resulting less-ionic molecules can associate and precipitate or form a gel as expected for an unbranched, uncharged glycan.

Carrageenans are mixtures of linear chains of nonuniform structures that have a negative charge due to numerous ionized sulfate half-ester groups along the chain (Section 3.3.10). These molecules do not precipitate at low pH because the sulfate group remains ionized at all practical pH values.

Solutions of gums are dispersions of hydrated molecules and/or aggregates of hydrated molecules. Their flow behavior is determined by the size, shape, ease of deformation (flexibility), and presence and magnitude of charges on these hydrated molecules and/or aggregates. There are two general kinds of flow exhibited by polysaccharide solutions: pseudoplastic (by far the most common) and thixotropic; both are characterized by shear thinning.

In pseudoplastic flow, a more rapid flow results from an increase in shear rate, that is, the greater the applied force, the less viscous it becomes (Figure 3.38). The applied force can be that of pouring, chewing, swallowing, pumping, mixing, or anything else that induces shear. The change in viscosity is independent of time, that is, the rate of flow changes instantaneously as the shear rate is changed.

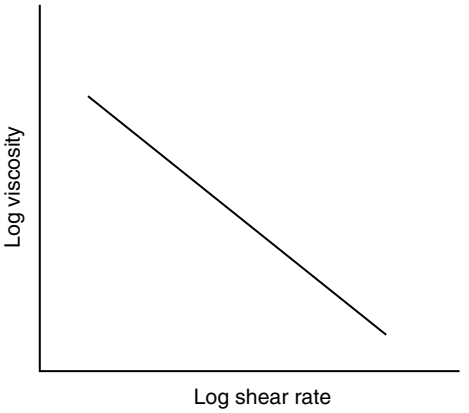

FIGURE 3.38 The logarithm of viscosity as a function of the shear rate for a pseudoplastic shear-thinning fluid.

In general, higher-molecular-weight gums form more pseudoplastic solutions. Certainly, stiffer, linear molecules produce the more pseudoplastic flow.

Gum solutions that are less pseudoplastic are said to give long flow;* such solutions are generally perceived as being slimy. More pseudoplastic solutions are described as having short flow and are generally perceived as being nonslimy. In food science, a slimy material is one that is thick, coats the mouth, and is difficult to swallow. Sliminess is inversely related to pseudoplasticity, that is, to be perceived as being nonslimy, there must be marked thinning at the low shear rates produced by chewing and swallowing.

Thixotropic flow is a second type of shear-thinning flow. In this case, the viscosity reduction that results from an increase in the rate of flow does not occur instantaneously. The viscosity of thixotropic solutions decreases under a constant rate of shear in a time-dependent manner and regains the original viscosity after cessation of shear, but again only after a clearly defined and measurable time interval. This behavior is due to a gel → solution → gel transition. In other words, a thixotropic solution at rest is a weak (pourable) gel (Section 3.3.4).

For solutions of most gums, an increase in temperature results in a decrease in viscosity. This loss of viscosity as the temperature is raised is often an important property, for it means that higher solids can be put into solution at a higher temperature; then the solution can be cooled for thickening. (Xanthan gum is an exception because the viscosity of its solutions is essentially constant at temperatures between 0°C and 100°C. See Section 3.3.9.)

3.3.4 Gels [12,13,26]

A gel is a continuous, three-dimensional network of connected molecules or particles (such as crystals, emulsion droplets, or molecular aggregates/fibrils) entrapping a large volume of a continuous liquid phase, much as does a sponge. In many food products, the gel network consists of polymer (polysaccharide and/or protein) molecules or fibrils formed from polymer molecules joined in junction zones by hydrogen bonding, hydrophobic associations (i.e., van der Waals attractions), ionic cross bridges, entanglements, or covalent bonds, and the liquid phase is an aqueous solution of low-molecular-weight solutes and portions of the polymer chains.

Gels have some characteristics of solids and some characteristics of liquids. When polymer molecules or fibrils formed from polymer molecules interact over portions of their lengths to form junction zones and a three-dimensional network (Figure 3.39), a fluid solution is changed into a material that can retain its shape (partially or entirely). The three-dimensional network structure offers sufficient resistance to an applied stress to cause it to behave in part as an elastic solid. However, the continuous liquid phase, in which molecules are completely mobile, makes a gel less stiff than an ordinary solid, causing it to behave in some respects as a viscous liquid. Therefore, a gel is a viscoelastic semisolid, that is, the behavior of a gel in response to an applied stress is partly that of an elastic solid and partly that of a viscous liquid.

Although gel-like or salve-like materials can be formed by high concentrations of particles (much like tomato paste), to form a gel from dissolved gum/hydrocolloid molecules, the polymer molecules or aggregates of molecules must partially come out of solution in junction zone regions to tie them together in a three-dimensional gel network structure. In general, if the junction zones grow after formation of the gel, the network becomes more compact, the structure contracts, and syneresis results. (The appearance of fluid droplets on the gel surface is called syneresis.)

* "Short flow" is exhibited by shear-thinning, primarily pseudoplastic, viscous solutions and "long flow" by viscous solutions that exhibit little or no shear-thinning. These terms were applied long before there were instruments to determine and measure rheological phenomena. They were arrived at in this way. When a gum or starch solution is allowed to drain from a pipette or a funnel, those that are not shear-thinning come out in long strings, while those that shear-thin form short drops. The latter occurs because as more and more fluid exits the orifice, the weight of the string becomes greater and greater, which causes it to flow faster and faster, and which causes it to shear-thin to the point that the string breaks into drops.

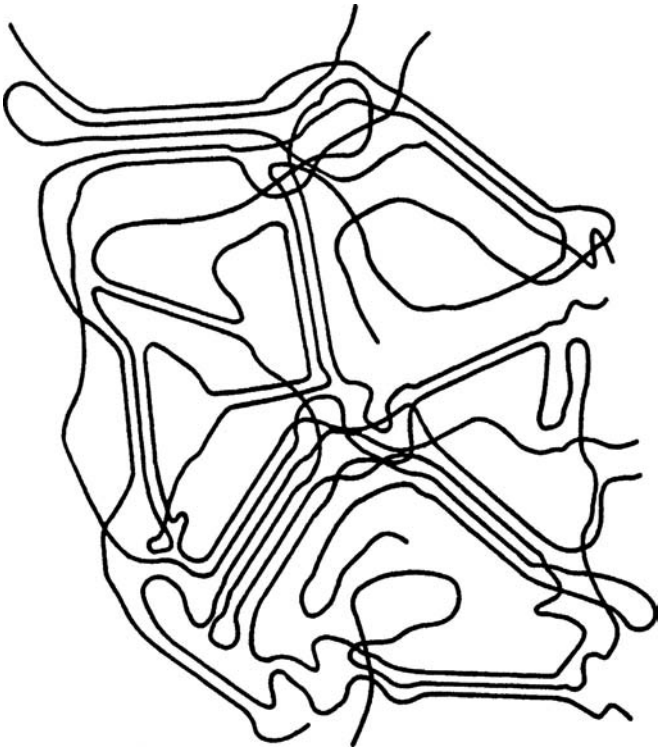

FIGURE 3.39 A diagrammatic representation of the type of three-dimensional network structure found in gels. Parallel side-by-side chains indicate the ordered, crystalline structure of a junction zone. The gaps between junction zones contain an aqueous solution of dissolved segments of polymer chains and other solutes.

Although polysaccharide gels generally contain no more than 1% polymer, that is, they may contain as much as 99% water, they can be quite strong. Examples of polysaccharide gels are dessert gels, aspics, structured fruit pieces, structured onion rings, meat-analog pet foods, jams, jellies, and confections such as gum drops.

Choice of a specific gum for a particular application depends on the viscosity or gel strength desired, the desired rheology, the pH of the system, temperatures during processing, interactions with other ingredients, the desired texture, and the cost of the amount needed to impart the desired properties. Consideration is also given to desired functional characteristics. These include a gum's ability to function as a binder, bodying agent, bulking agent, crystallization inhibitor, clarifying agent, cloud agent, coating agent/film former, emulsifier, emulsion stabilizer, encapsulating agent, fat mimetic, flocculating agent, foam stabilizer, mold release agent, suspension stabilizer, swelling agent, syneresis inhibitor, and whipping agent and its ability to effect water absorption and binding (water retention and migration control). Each food gum tends to have an outstanding property (perhaps several unique properties), which is often the basis for its choice for a particular application (Table 3.5).

3.3.5 Polysaccharide Hydrolysis

Polysaccharides are relatively less stable to hydrolytic cleavage than are proteins and may, at times, undergo depolymerization during food processing and/or storage of foods.* Often, food gums are

* On the other hand, polysaccharides do not undergo denaturation.

TABLE 3.5
Predominantly Used, Water-Soluble, Nonstarch Food Polysaccharides

Gum	Source	Class	General Shape	Monomer Units and Linkages (Approx. Ratios)	Noncarbohydrate Substituent Groups	Water Solubility	Key General Characteristics	Major Food Applications
Algins (alginates) (generally sodium alginate)	Brown algae	Seaweed (algal) extract Poly(uronic acid)	Linear	Block copolymer of the following units: →4)-βMan*p*A (1.0) →4)-αLGul*p*A (0.5–2.5)		Sodium alginate soluble	Gels with Ca^{2+} Viscous, not very pseudoplastic solutions	Forms nonmelting gels (dessert gels, fruit analogs, other structured foods) Meat analogs
						Alginic acid insoluble		Alginic acid forms soft, thixotropic, nonmelting gels (tomato aspic, jelly-type bakery fillings, filled fruit-containing breakfast cereal products)
					Hydroxypropyl ester groups in propylene glycol alginate (PGA)	Soluble	Surface active Solutions stable to acids and Ca^{2+}	Emulsion stabilization in creamy salad dressings Thickener in low-calorie salad dressings
Carboxymethyl-cellulose (CMC)	Derived from cellulose	Modified cellulose	Linear	→4)-βGlc*p*-(1→	Carboxymethyl ether (DS 0.4–0.8)[a]	High	Clear, stable solutions that can be either pseudoplastic or thixotropic	Retards ice crystal growth in ice creams and other frozen dessert products Thickener, suspending aid, protective colloid, and improver of mouthfeel, body, and texture in a variety of dressings, sauces, and spreads Lubricant, film former, and processing aid for extruded products Batter thickener and humectant in cake and related mixes Moisture binder and retarder of crystallization and/or syneresis in icings, frostings, toppings, fillings, and puddings Syrup thickener Suspending aid and thickener in dry powder, hot and cold drink mixes Gravy maker in dry pet food

(Continued)

TABLE 3.5
(Continued)

Gum	Source	Class	General Shape	Monomer Units and Linkages (Approx. Ratios)	Noncarbohydrate Substituent Groups	Water Solubility	Key General Characteristics	Major Food Applications
Carrageenans	Red algae	Seaweed (algal) extracts Sulfated galactans	Linear		Sulfate half-ester			Secondary stabilizer in ice cream and related products Preparation of evaporated milk, infant formulas, freeze–thaw stable whipped cream, dairy desserts, and chocolate milk Meat coating Improves adhesion and increases water-holding capacity of meat emulsion products Improves texture and quality of low-fat meat products
				κ types: $\rightarrow$3)-βGal*p* 4-SO_3^- (1$\rightarrow$4)-3,6An-αGal*p* (1$\rightarrow$		κ types: Na^+ salt soluble in cold water, K^+ and Ca^{2+} salts insoluble; all salts soluble at temperatures > 65°C; soluble in hot milk, insoluble in cold milk	Forms stiff, brittle, thermoreversible gels with $K^+ > Ca^{2+}$; thickens and gels milk at low concentration; synergistic gelation with LBG	

				ι types: →3)-β-Gal*p* 4-SO_3^- (1→4)-3,6An-α-Gal*p* 2-SO_3^-(1→		ι types: Na^+ salt soluble in cold water, K^+ and Ca^{2+} salts insoluble; all salts soluble at temperatures > 55°C; soluble in hot milk, insoluble in cold milk	Forms soft, resilient, thermoreversible gels with $Ca^{2+} > K^+$; gels do not synerese and have good freeze–thaw stability	
				λ types: →3)-β-Gal*p* 2-SO_3^- (1→4)-α-Gal*p* 2,6-diSO_3^-(1→		λ types: all salts soluble in hot and cold water and milk	Thickens cold milk	
Curdlan	Fermentation medium	Microbial polysaccharide	Linear	→3)-β-Glc*p*-(1→		Soluble	Gels irreversibly upon heating of solutions	Layered, nonmelting dessert gels
Gellan	Fermentation medium	Microbial polysaccharide	Linear	→4)-α-LRha*p*-(1→3)-β-Glc*p*-(1→4)-β-Glc*p*A-(1→4)-β-Glc*p*-(1→	Native type contains an acetate and a glycerate ester group on each repeating unit	Soluble in warm water	Gels with any cation Solutions have high yield values Low-acyl types form firm, brittle, nonelastic gels High-acyl types form soft, elastic, nonbrittle gels	Bakery mixes Nutrition bars Nutritional beverages Fruit toppings Sour cream and yogurt products

(Continued)

TABLE 3.5
(Continued)

Gum	Source	Class	General Shape	Monomer Units and Linkages (Approx. Ratios)	Noncarbohydrate Substituent Groups	Water Solubility	Key General Characteristics	Major Food Applications
Guar gum	Guar seed	Seed galacto-mannan	Linear with single-unit branches (behaves as a linear polymer)	→4)-βMan$p$(~0.56) αGal$p$ 1 ↓ 6 →4)-βMan$p$(~1.0) (Man:Gal = ~ 1.56:1)		High	Stable, opaque, very viscous, moderately pseudoplastic solutions Economical thickening	Binds water, prevents ice crystal growth, improves mouthfeel, softens texture produced by carrageenan + LBG, and slows meltdown in ice cream and ices Dairy products, prepared meals, bakery products, sauces, pet food
Gum arabic (gum acacia)	Acacia tree	Exudate gum	Branch-on-branch, highly branched	Complex, variable structure, contains polypeptide		Very high	Emulsifier and emulsion stabilizer Compatible with high concentrations of sugar Very low viscosity at high concentrations	Prevents sucrose crystallization in confections Emulsifies and distributes fatty components in confections Preparation of flavor oil-in-water emulsions Component of coating of pan-coated candies Preparation of flavor powders
Inulin	Chicory root	Plant extract	Linear	→2)-βFru$f$(1→		Soluble	Gels when hot solutions are cooled Can be used as a fat mimetic	Ingredient in nutrition, breakfast, and energy bars and vegetable patties as a source of dietary fiber and fat mimetic
Locust bean gum (carob gum, LBG)	Locust bean (carob) seed	Seed galacto-mannan	Linear with single-unit branches (behaves as a linear polymer)	→4)-βMan$p$(~2.5) αGal$p$ 1 ↓ 6 →4)-βMan$p$(~1.0) (Man:Gal = ~ 3.5:1)		Soluble only in hot water; requires 90°C for complete solubilization	Interacts with xanthan and carrageenan to form rigid gels; rarely used alone	Provides excellent heat shock resistance, smooth meltdown, and desirable texture in ice creams and other frozen dessert products

Methylcelluloses (MC) and hydroxy-propylmethyl-celluloses (HPMC)	Derived from cellulose	Modified cellulose	Linear	→4)-βGlc*p*-(1→	Hydroxypropyl (MS 0.02–0.3)[a] and methyl (DS 1.1–2.2)[a] ether groups	Soluble in cold water; insoluble in hot water	Clear solutions that are thermal gelling; surface active	MC: Provides fat-like characteristics Reduces fat absorption in fried products Imparts creaminess through film and viscosity formation Provides lubricity Gas retention during baking Moisture retention and control of moisture distribution in bakery products (increases shelf life and imparts tenderness) HPMC: Nondairy whipped toppings, where it stabilizes foams, improves whipping characteristics, prevents phase separation, and provides freeze–thaw stability
Pectins	Citrus peel Apple pomace	Plant extract Poly(uronic acid)	Linear	Primarily composed of →4)-αGal*p*A units	Methyl ester groups May contain amide groups	Soluble	Forms jelly- and jam-type gels in presence of sugar and acid or with Ca^{2+}	HM pectin: high-sugar jellies, jams, preserves, and marmalades Acidic milk drinks LM pectin: dietetic jellies, jams, preserves, and marmalades
Xanthan	Fermentation medium	Microbial polysaccharide	Linear with trisaccharide unit; branches on every other main chain unit (behaves as a linear polymer)	βMan*p* 1 ↓ 4 βGlc*p* A 1 ↓ 2 αMan*p* 6-Ac 1 ↓ 3 →4)-βGlc*p*-(1→4)-βGlc*p*-(1→	Acetyl ester Pyruvyl cyclic acetal on some βMan*p* end units	High	Very pseudoplastic, high viscosity solutions; excellent emulsion and suspension stabilizer; solution viscosity unaffected by temperature; solution viscosity unaffected by pH; excellent salt compatibility; synergistic increase in viscosity upon interaction with guar gum; heat reversible gelation with LBG	Stabilization of dispersions, suspensions, and emulsions Thickener

[a] For definitions of DS and MS (see Sections 3.3.6.10 and 3.4.3).

deliberately depolymerized. One reason why food gums would be deliberately depolymerized is so that a relatively high concentration can be used to provide body (*mouthfeel*) without producing undesirable viscosity.

Hydrolysis of glycosidic bonds joining monosaccharide (glycosyl) units in oligo- and polysaccharides can be catalyzed by acids (H^+) and/or enzymes. The extent of depolymerization, which has the effect of reducing viscosity, is determined by the pH (acid), temperature, time at that temperature and pH, and structure of the polysaccharide. Hydrolysis occurs most readily during thermal processing of acidic foods (as opposed to storage) because of the elevated temperature. Defects associated with depolymerization during processing can usually be overcome by using more of the polysaccharide (gum) in the formulation to compensate for breakdown, using a higher-viscosity grade of the gum, again to compensate for any depolymerization, or using a relatively more acid-stable gum. Depolymerization can also be an important determinant of shelf life.

Polysaccharides are also subject to enzyme-catalyzed hydrolysis. The rate and end products of this process are controlled by the specificity of the enzyme, pH, temperature, and time. Polysaccharides, like any and all other carbohydrates, are subject to microbial attack because of their susceptibility to enzyme-catalyzed hydrolysis. Furthermore, gum products are very seldom, if ever, delivered sterile, a fact that must be considered when using them as ingredients.

3.3.6 Starch [66,68]

The unique chemical and physical characteristics and nutritional aspects of starch set it apart from all other carbohydrates. Starch is the predominant food reserve substance in plants and provides 70–80% of the calories consumed by humans worldwide. Starch and starch hydrolysis products constitute most of the digestible carbohydrate in the human diet. Also, the amount of starch used in the preparation of food products—without counting that present in flours used to make bread and other bakery products, that naturally occurring in grains used to make breakfast cereals, or that naturally consumed in fruits and vegetables—greatly exceeds the combined use of all other food hydrocolloids.

Commercial starches are obtained from cereal grain seeds, particularly from normal corn, waxy corn (waxy maize), high-amylose corn, wheat, and various rices, and from tubers and roots, particularly potato and cassava (tapioca starch). Starches and modified starches have an enormous number of food uses, including adhesive, binding, clouding, dusting, film forming, foam strengthening, gelling, glazing, moisture retaining, stabilizing, texturizing, and thickening applications.

Starch is unique among carbohydrates because it occurs in nature as discrete particles called granules. Starch granules are insoluble; they hydrate only slightly in cold water. As a result, they can be dispersed in water, producing low-viscosity slurries that can be easily mixed and pumped, even at concentrations greater than 35%. The viscosity building (thickening) power of starch is realized only when a slurry of granules is cooked. Heating a 5% slurry of most unmodified starch granules to about 80°C (175 F) with stirring produces a very high viscosity dispersion called a paste. A second uniqueness is that most starch granules are composed of a mixture of two polymers: an essentially linear polysaccharide called amylose and a highly branched polysaccharide called amylopectin.

3.3.6.1 Amylose

While amylose is essentially a linear chain of (1→4)-linked α-D-glucopyranosyl units, many amylose molecules contain a few branches connected by α-D-(1→6) linkages at the branch points. Perhaps, 1 in 180–320 units, or 0.3–0.5% of the linkages, are branch points. The branches in branched amylose molecules are either very long or very short, and most branch points are separated by large distances so that the physical properties of amylose molecules are essentially those of linear molecules. Amylose molecules have molecular weights that are, on average, about 10^6.

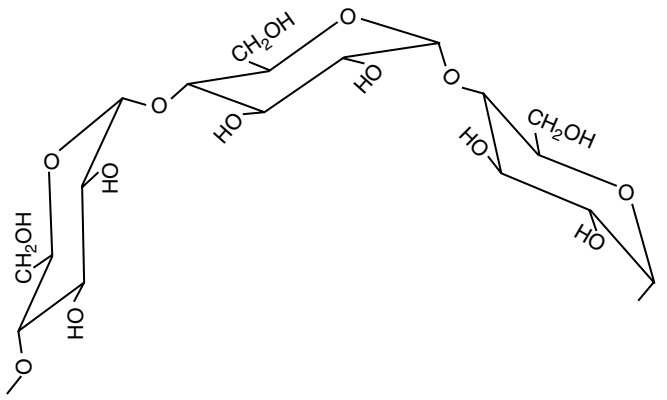

FIGURE 3.40 A trisaccharide segment of an unbranched portion of amylose or amylopectin molecule.

TABLE 3.6
General Properties of Some Starch Granules and Their Pastes

	Common Corn Starch	Waxy Maize Starch	High-Amylose Corn Starch	Potato Starch	Tapioca Starch	Wheat Starch
Granule size (major axis, μm)	2–30	2–30	2–24	5–100	4–35	2–55
% Amylose	28	<2	50–70	21	17	28
Gelatinization/pasting temp. (°C)[a]	62–80	63–72	66–170[b]	58–65	52–65	52–85
Relative viscosity	Medium	Medium high	Very low[b]	Very high	High	Low
Paste rheology[c]	Short	Long	Short	Very long	Long	Short
Paste clarity	Opaque	Very slightly cloudy	Opaque	Clear	Clear	Opaque
Tendency to gel/retrograde	High	Very low	Very high	Medium to low	Medium	High
Lipid (% DS)	0.8	0.2	—	0.1	0.1	0.9
Protein (% DS)	0.35	0.25	0.5	0.1	0.1	0.4
Phosphorus (% DS)	0.00	0.00	0.00	0.08	0.00	0.00
Flavor	Cereal (slight)	"Clean"		Slight	Bland	Cereal (slight)

[a] From the initial temperature of gelatinization to complete pasting.
[b] Under ordinary cooking conditions, where the slurry is heated to 95–100°C, high-amylose corn starch produces essentially no viscosity. Pasting does not occur until the temperature reaches 160–170°C (320–340 F).
[c] For a description of long and short flow (see Section 3.3.3).

The axial → equatorial position coupling of the (1→4)-linked α-D-glucopyranosyl units in amylose chains gives the molecules a right-handed spiral or helical shape (Figure 3.40). The interior of the helix contains a predominance of hydrogen atoms and is hydrophobic/lipophilic, while the hydroxyl groups are positioned on the exterior of the coil. Looking down the axis of the helix gives a view very much like that of looking down a stack of α-cyclodextrin molecules (Section 3.2.4) because each turn of the helix contains about 6 α-D-glucopyranosyl units linked (1→4).

Most starches contain about 25% amylose (Table 3.6). The two high-amylose corn starches that are commercially available have apparent amylose contents of about 52% and 70–75%.

3.3.6.2 Amylopectin [39]

Amylopectin, is a very large, very highly branched molecule, with branch point linkages constituting 4–5% of the total linkages. Amylopectin consists of a chain containing the only reducing end group, to which are attached numerous branch chains, to which are attached one to several third layer branch chains. The branches of amylopectin molecules are clustered (Figure 3.41) and

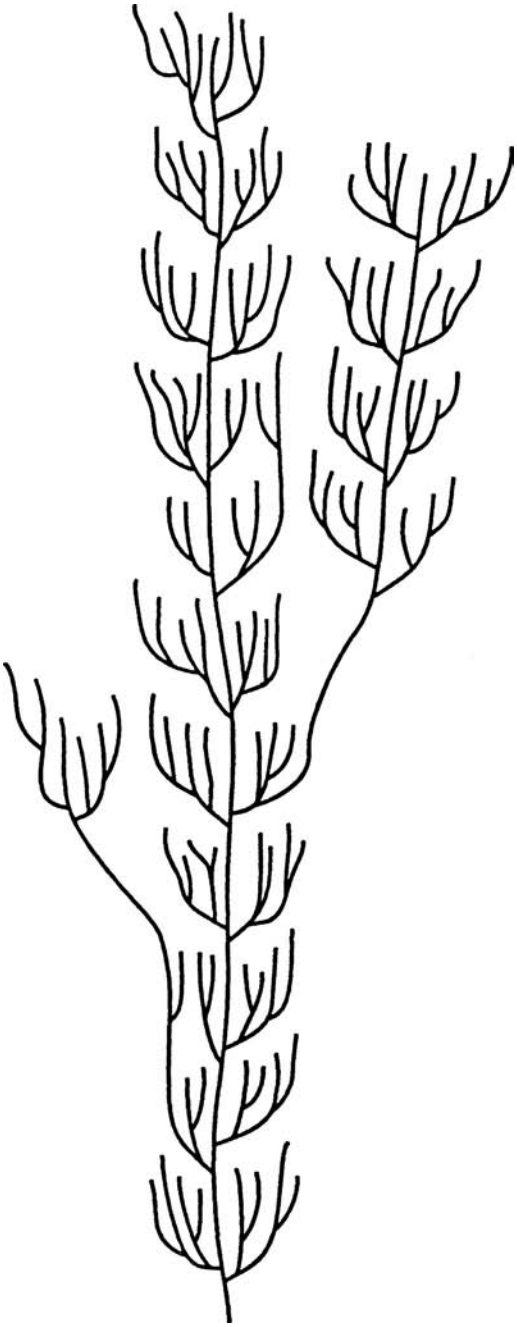

FIGURE 3.41 A diagrammatic representation of a portion of an amylopectin molecule.

occur as double helices. Molecular weights from 10^7 (DP ~60,000) up to perhaps 5×10^8 (DP ~3,000,000) make amylopectin molecules among the largest, if not the largest, molecules found in nature.

Amylopectin is present in all starches. It constitutes about 75% of most common starches (Table 3.6). Some starches consist entirely of amylopectin and are called waxy or amylopectin starches. Waxy corn (waxy maize), the first grain recognized as one in which the starch consists only of amylopectin, is so termed because, when the kernel is cut, the new surface has a vitreous or waxy appearance. Most other all-amylopectin starches are also called waxy although, as in corn, there is no wax present.

Potato amylopectin is unique among the commercial starches in having more than trace amounts of phosphate ester groups. These phosphate ester groups are attached most often (60–70%) at an O-6 position, with the other third at O-3 positions. These phosphate ester groups occur about once in every 215–560 α-D-glucopyranosyl units.

3.3.6.3 Starch Granules [72]

Starch granules are made up of amylose and/or amylopectin molecules arranged radially. They contain both crystalline and noncrystalline regions in alternating layers.* The clustered branches of amylopectin occur as packed double helices. The packing together of these double-helical structures forms small crystalline lamellae. The more dense layers of starch granules, which alternate with less dense amorphous layers, contain greater amounts of the crystalline lamellae. The radial, ordered arrangement of starch molecules in a granule is evident from the birefringence of granules, evidence for which is the polarization cross (white cross on a black background) seen in a polarizing microscope with the polarizers set at 90° to each other. The center of the cross is at the hilum, the origin of growth of the granule.

Corn starch granules, even from a single source, have mixed shapes, some being almost spherical, some angular, and some indented (for the size, see Table 3.6). Wheat starch granules are lenticular and have a bimodal size distribution (roughly <10 and >10 μm), with the larger granules being lenticular in shape. Rice granules are the smallest of the commercial starch granules (1–9 μm), although the small granules of wheat starch are almost the same size. Many of the granules in tuber and root starches, such as potato and tapioca starches, tend to be larger than those of seed starches and are generally less dense and easier to cook. Potato starch granules may be as large as 100 μm along the major axis.

All commercial starches contain small amounts of ash, lipid, and protein (Table 3.6). The phosphorus content of potato starch (0.06–0.1%, 600–1000 ppm) is due to the presence of the phosphate ester groups on amylopectin molecules. The phosphate ester groups give potato starch amylopectin a slight negative charge, resulting in some Coulombic repulsion that may contribute to the rapid swelling of potato starch granules in warm water and to several properties of potato starch pastes, namely, their high viscosities, good clarity (Table 3.6), and low rate of retrogradation (Section 3.3.6.7). Cereal starch molecules either do not have phosphate ester groups or have much smaller amounts than do potato starch molecules. Only the cereal starches contain endogenous lipids in the granules. These internal lipids are primarily FFA and lysophospholipid (LPL), largely lysophosphatidyl choline (89% in corn starch), with the ratio of FFA to LPL varying from one cereal starch to another.

3.3.6.4 Granule Gelatinization and Pasting [6,52]

Undamaged starch granules are insoluble in cold water, but can imbibe water reversibly, that is, they can swell slightly, and then return to their original size on drying. When heated in water,

* Starch granules are composed of layers somewhat like the layers of an onion, except that the layers cannot be peeled off.

starch granules undergo a process called gelatinization. Gelatinization is the disruption of molecular order within granules. Evidence for the loss of order includes irreversible granule swelling, loss of birefringence, and loss of crystallinity. Leaching of amylose occurs during gelatinization, but some leaching of amylose can also occur prior to gelatinization. Total gelatinization of a population of granules occurs over a temperature range (Table 3.6). The apparent temperature of initial gelatinization and the range over which gelatinization occurs depends on the method of measurement and on the starch:water ratio, granule type, and the degree of heterogeneity within the granule population under observation. (All populations of starch granules are heterogenous.) Several aspects of gelatinization of a population of granules can be determined. These are the initiation temperature, the midpoint temperature, and the completion temperature.

Continued heating of starch granules in excess water results in further granule swelling, additional leaching of soluble components (primarily amylose), and eventually, especially with the application of shear forces, total disruption of granules. These phenomena result in the formation of a starch paste. (In starch technology, what is called a paste is what results from heating a starch slurry.) Granule swelling and disruption produces a viscous mass (the paste) consisting of a continuous phase of solubilized amylose and/or amylopectin molecules and a discontinuous phase of granule remnants (granule ghosts* and fragments). Complete molecular dispersion is not accomplished except, perhaps, under conditions of high temperature, high shear, and excess water—conditions that are seldom, if ever, encountered in the preparation of food products. Cooling of a hot, normal corn starch paste results in a viscoelastic, firm, rigid gel.

Because gelatinization of starch is an endothermic process, differential scanning calorimetry (DSC), which measures both the temperature and the enthalpies of gelatinization, is widely used to follow the process. Although there is no complete agreement on the interpretation of DSC data and the events that take place during gelatinization of starch granules, the following general picture is widely accepted. Water acts as a plasticizer. Its mobility-enhancing effect is first realized in the amorphous regions, which physically have the nature of a glass. When starch granules are heated in the presence of sufficient water (at least 60%) and a specific temperature (T_g, the glass transition temperature) is reached, the plasticized amorphous regions of the granules undergo a phase transition from a glassy state to a rubbery state.† However, the peak for absorption of energy associated with this transition is not often, if ever, seen by DSC because the regions of crystallinity, that is, the ordered, packed, double-helical branches of amylopectin, are contiguous and connected by covalent bonds to the amorphous regions and melting of the crystallites immediately follows the glass transition. Because the enthalpy of initial melting (T_m) is so much larger than that of the glass transition, the latter is usually not evident.

Melting of lipid–amylose complexes occurs at much higher temperatures (100–120°C in excess water) than does melting of the amylopectin double-helical branches packed in crystalline order. Lipid–amylose complexes are made with single-helical segments of amylose molecules when a starch paste containing fatty acids or monoacyl glycerolipids is cooled. A DSC peak for this event is absent in waxy starches (without amylose).

Under normal food processing conditions (heat and moisture, although many food systems contain limited water as far as starch cooking is concerned), starch granules quickly swell beyond the reversible point. Water molecules enter between chains, break interchain bonds, and establish hydration layers around the separated molecules. This plasticizes (lubricates) chains, so they become more fully separated and solvated. Entrance of large amounts of water causes granules to swell to several times their original size. When a 5% starch suspension is gently stirred and heated, granules imbibe water until much of the water is absorbed by them, forcing them to swell, press against

* The granule ghost is the remnant remaining after cooking under no shear to moderate shear. It consists of the outer portion of the granule. It appears as an insoluble, outer envelope.

† A glass is a mechanical solid (a supercooled liquid) capable of supporting its own weight against flow. A rubber is an undercooled liquid that can exhibit viscous flow (see Chapter 2 for further details).

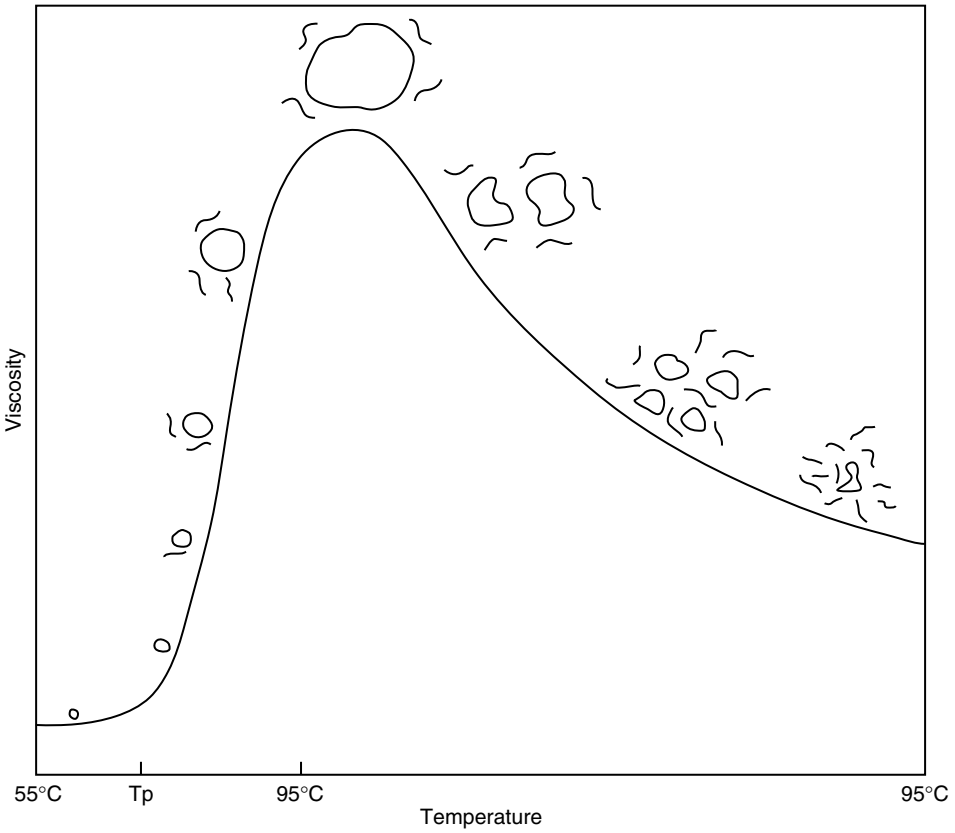

FIGURE 3.42 Representative cooking/pasting curve showing viscosity changes related to typical starch granule swelling and disintegration as a granule suspension is heated to 95°C and then held at that temperature using an instrument that imparts low shear.

each other, and fill the container with a highly viscous starch paste with most of the water inside the swollen granules. Such a starch paste has viscosity like that of a pudding because most of the space is composed of swollen granules that move past one another only with great difficulty. Such highly swollen granules of native starches are easily broken and disintegrated by stirring, resulting in a decrease in viscosity. As starch granules swell, hydrated amylose molecules diffuse through the mass to the external phase (water), a phenomenon responsible for some aspects of paste behavior. Results of starch swelling can be recorded using various instruments that record the viscosity continuously as the temperature is increased, held constant for a time, and then decreased (Figure 3.42).

Most suspensions of starch granules are stirred while being heated to prevent the granules from settling to the bottom of the container. Instruments that record changes that occur during starch pasting and paste behavior as a function of temperature to produce curves like those in Figure 3.42 also employ stirring. By the time peak viscosity is reached, some granules have been broken by stirring. With continued stirring, more granules rupture and fragment, causing a further decrease in viscosity. On cooling, some starch molecules partially reassociate to form a precipitate or gel. This process is called retrogradation (Section 3.3.6.7). The firmness of the gel depends on the extent of junction zone formation (Section 3.3.4). Junction zone formation is influenced (either facilitated or hindered) by the presence of other ingredients such as fats, proteins, sugars, and acids and the amount of water present.

3.3.6.5 Uses of Unmodified Starches

Starches serve a variety of roles in food production. Principally they are used to produce desired texture qualities (Section 3.3.6.9). Primarily, they provide body and bulk. The extent of starch gelatinization in baked goods strongly affects product properties, including storage behavior and rate of digestion. In baked products made from low-moisture doughs, many wheat starch granules remain ungelatinized. In higher-moisture products, most or all of the granules become gelatinized.

Most starch used as a food ingredient is "modified food starch" (Section 3.3.6.10) because, for the most part, textures of cooked suspensions of native starches, especially of native normal corn starch, are undesirable. The clear, cohesive pastes produced from waxy maize starch are somewhat more desirable, but even waxy maize starch is usually chemically modified to improve the functionalities it imparts. Unmodified potato starch is used in extruded cereal and snack food products and in dry mixes for soups and cakes. Rice starch produces opaque gels useful for baby food. Waxy rice starch gels are clear and cohesive. Wheat starch gels are weak and have a slight flavor due to residual flour components. Tuber (potato) and root (tapioca) starches have weak intermolecular bonding and swell greatly to give high-viscosity pastes (Table 3.6), but if shear is applied, the viscosity decreases quickly because the highly swollen granules break easily.

3.3.6.6 Starch Gelatinization within Vegetable Tissues [1,29,30,45]

The majority of dietary starch occurs within grain- and vegetable-based food products that contain starch as the predominant dry matter. Thus, it is important to understand the thermal properties of starch within these native environments as it relates to acceptability and texture of processed foods. The degree to which starch is gelatinized within food systems is generally dependent upon both the amount of water present and the extent of the heat treatment. As already mentioned, in some baked products, starch granules may remain ungelatinized even when heated to high temperatures. In pie crust and some cookies that are high in fat and low in moisture, about 90% of the wheat starch granules remain ungelatinized. In bread and cakes, which have higher moisture contents, about 96% of the wheat starch granules are gelatinized, but because they are heated without shear, the granules are still evident and can be isolated, although many are deformed.

Thermal processing (blanching, baking, boiling, steaming, frying) of vegetables is generally sufficient to induce a desired tissue softening. Following a heating process, vegetable tissue becomes more susceptible to fracture between (as opposed to through) its parenchyma cells.

Parenchyma tissue is the most abundant type of tissue in edible plants. In general, parenchyma tissue is comprised of aggregates of polygonally-shaped cells, each of which contains clusters of starch granules surrounded by a cellulosic cell wall. Adjoining cells are attached or cemented together by a middle lamella, which consists primarily of pectic substances. Water, which is the predominant constituent of most vegetable tissues, resides primarily in vacuoles within cells (84%), while the balance is associated with starch granules (13%) and cell wall components (3%).

As plant tissue is heated, the semicrystalline starch granules take up available water within cells and undergo swelling and gelatinization (Figure 3.43). The native moisture within parenchyma tissue is generally sufficient to plasticize starch granules and facilitate gelatinization, though the temperature at which these thermal events occur is slightly higher for starch granules housed within native plant cells compared to isolated starch. The higher gelatinization temperature of *in situ* starch has been attributed to the presence of solutes. Though starch gelatinization is complete within plant tissue (molecular order is fully lost), granule swelling is limited by the boundaries of the surrounding cell walls. Starch granules swell (with some leaching of amylose from cells) to fill most of the entire volume of their respective cells, producing a swollen starch mass that may still possess some subtly discernable granule remnants. Granule swelling during heating has been shown to exert an observable internal pressure on parenchyma cell walls (estimated at 100 kPa). Though the magnitude of swelling pressure is itself insufficient to bring about cell rupture (cells generally remain

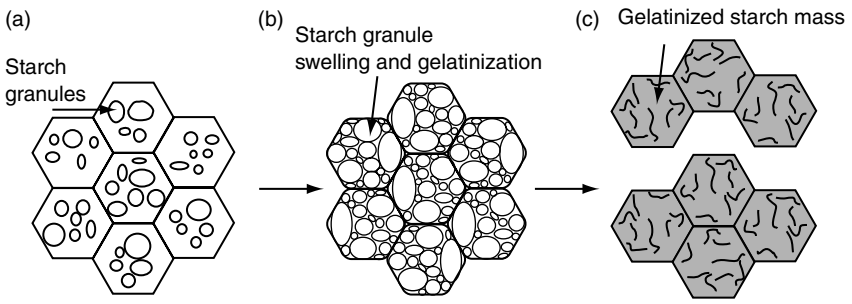

FIGURE 3.43 Within plant parenchyma tissue, starch granules (a) within cells undergo swelling and gelatinization during heating to exert a temporary "swelling pressure" on surrounding cell walls (b). With further heating, starch granules evolve into a fairly uniform gelatinized starch mass within cells (c). Heated tissue becomes prone to increased sloughing (cell separation), which is primarily attributable pectin degradation within the middle lamella, though starch swelling pressure is thought to contribute a significant secondary role.

intact), isolated potato parenchyma cells temporarily increase in size and become more spherical as a result of starch gelatinization. This phenomenon, referred to as cell "rounding off," occurs in concert with pectin degradation by β-elimination within the middle lamellae to cause softening of the parenchyma tissue. As the characteristic softening phenomenon is observed in tissues that do not contain appreciable starch contents, such as tomatoes, this effect is primarily attributed to pectin degradation within the middle lamellae.

Nevertheless, in starch-containing tissues, such as potato, a high starch content and/or degree of starch granule swelling is associated with a softer, more friable cooked tissue. It is thought that the cell "rounding off" phenomenon exerts physical pressure on a partially degraded or weakened middle lamellae, contributing secondarily to cell separation or tissue sloughing. Also, the degree to which gelatinized starch swells to fill the volume of parenchyma cells is thought to influence the human perception of tissue moistness in the mouth. A high starch content and swelling capacity is generally more effective in binding up free moisture within the cooked tissue, producing a corresponding dry mouthfeel. Cooked potato texture has been traditionally classified in terms of "mealiness" and "waxiness." A mealy texture is characterized by a dry-appearing tissue that crumbles or sloughs easily. In contrast, a waxy tissue (not to be confused with a waxy starch) is defined by a moist appearance, a gummy mouthfeel, and a firm texture. Generally, mealy potatoes are deemed more suitable for the majority of processed products (French fries, mashed potatoes, etc.). Waxy potato varieties find application in boiling and canning. In conclusion, starch gelatinization behavior appears to exert significant influence on cooked vegetable texture and end-use potential through its secondary role in tissue softening (cell "rounding off") and its water-binding capabilities within parenchyma tissue.

3.3.6.7 Retrogradation and Staling [23,42,43,52]

As already pointed out, cooling a hot starch paste generally produces a firm, viscoelastic gel. The formation of the junction zones of a gel can be considered to be the first stage of an attempt by starch molecules to crystallize. As starch pastes are cooled and stored, the starch becomes progressively less soluble. In dilute solution, starch molecules will precipitate. The collective processes of the starch molecules in a solution or paste becoming less soluble is called retrogradation. Retrogradation of cooked starch involves both of the two constituent polymers, amylose and amylopectin, with amylose undergoing retrogradation at a much more rapid rate than amylopectin. The rate of retrogradation depends on several variables, including the molecular ratio of amylose to amylopectin; structures of the amylose and amylopectin molecules, which is determined by the botanical source of the starch; temperature; starch concentration; and presence and concentration of other ingredients, primarily surfactants and salts. Many quality defects in food products, such as bread staling and loss of viscosity and precipitate formation in soups and sauces, are due, at least in part, to starch retrogradation.

Staling of baked goods is noted by an increase in crumb firmness and a loss in the perception of product freshness. Staling begins as soon as baking is complete and the product begins to cool. The rate of staling is dependent on the product formulation, the baking process, and storage conditions. Staling is due, at least in part, to the gradual transition of amorphous starch to a partially crystalline, retrograded state. In baked goods, where there is just enough moisture to gelatinize starch granules (while retaining a granule identity), amylose retrogradation (insolubilization) may be largely complete by the time the product has cooled to room temperature. Retrogradation of amylopectin is believed to involve primarily association of its outer branches and requires a much longer time than amylose retrogradation, giving it prominence in the staling process that occurs with time after the product has cooled.

Most polar lipids with surfactant properties retard crumb firming by forming complexes with starch polymer molecules. Compounds such as glyceryl monopalmitate (GMP), other monoglycerides and their derivatives, and sodium stearoyl 2-lactylate (SSL) are incorporated into doughs of bread and other baked goods, in part to increase shelf life.

3.3.6.8 Starch Complexes [7]

Because amylose chains are helical with hydrophobic (lipophilic) interiors, they are able to form complexes with linear hydrophobic portions of molecules that can fit in the hydrophobic tube. Iodine (as I_3^-) complexes with both amylose and amylopectin molecules. Again, the complexing occurs within the hydrophobic interior of the helical segments. With amylose, the long helical segments allow long chains of poly(I_3^-) to form and produce the blue color used as a diagnostic test for starch. The amylose–iodine complex contains 19% iodine and determination of the amount of complexing can be used to measure the amount of apparent amylose present in a starch. Amylopectin forms a reddish-purple color with iodine because the branch chains of amylopectin are too short for formation of long chains of poly(I_3^-).

Polar lipids (surfactants/emulsifiers and fatty acids) can affect starch pastes and starch-based foods in one or more of three ways as a result of complex formation: (1) by affecting the processes associated with starch gelatinization and pasting (i.e., the loss of birefringence, granular swelling, leaching of amylose, melting of the crystalline regions of starch granules, and viscosity increases during cooking), (2) by modifying the rheological behavior of the resulting pastes, and (3) by inhibiting the crystallization of starch molecules associated with the retrogradation process. Here too, complexation with emulsifiers occurs much more readily with, and has a much greater effect on, the amylose component than on the amylopectin component, so emulsifiers affect normal starches much more than waxy maize starch.

Certain flavor and aroma compounds also complex with starch, resulting in reduced perception in starchy foods. The binding of such compounds to starch, primarily amylose, molecules appears to be complex with competitive, synergistic, and antagonistic effects. However, the major reason all polysaccharides (starches and food gums) reduce the perception of flavors and aromas is a limitation of diffusion of flavor molecules to taste buds and aroma molecules to the surface where they can escape due to the increased viscosity starches and gums impart. Which of these processes and specific changes occur depends on the structure of the polar lipid, the starch employed, and the product to which it is added.

3.3.6.9 Hydrolysis of Starch [51,61]

Starch molecules, like all other polysaccharide molecules, are depolymerized by hot acids. Hydrolysis of the glycosidic bonds occurs more or less randomly to produce, initially, very large fragments. Commercially, hydrochloric acid is sprayed onto well-mixed starch, or stirred slightly moistened, granular starch is treated with hydrogen chloride gas, and the mixture is then heated until the desired degree of depolymerization is obtained.

TABLE 3.7
Functional Properties of Starch Hydrolysis Products

Properties Enhanced by Greater Hydrolysis[a]	Properties Enhanced in Products of Less Conversion[b]
Sweetness	Ability to produce viscosity
Hygroscopicity and humectancy	Ability to provide body
Freezing point depression	Foam stabilization
Flavor enhancement	Ice crystal growth prevention
Fermentability	Sugar crystallization prevention
Browning reaction	

[a] High-conversion (high-DE) syrups.
[b] Low-DE syrups and maltodextrins.

The acid is neutralized, and the product is recovered, washed, and dried. The products are still granular, but break up (cook out) much more easily than does the parent untreated starch. They are called acid-modified, thin-boiling, or thinned starches. Even though only a few glycosidic bonds are hydrolyzed, the starch granules disintegrate much more easily during heating in water. Acid-modified starches form gels with improved clarity and increased strength, even though they provide less solution viscosity. Thin-boiling starches are used as film formers and adhesives in products such as pan-coated nuts and candies and whenever a strong gel is desired, for example, in gum candies (such as jelly beans, jujubes, orange slices, and spearmint leaves) and in processed cheese loaves. To prepare products that form especially strong and fast-setting gels, a high-amylose corn starch is used as the base starch. The functional properties of the hydrolysis products of starch are given in Table 3.7.

More extensive depolymerization of granular starch with acid produces dextrins. Dextrins produce lower viscosities at equal concentrations than do thin-boiling starches and can be used at high concentrations in food processing. They have film forming and adhesive properties and are useful in products such as pan-coated, roasted nuts and candies. They are also used as fillers, encapsulating agents, and carriers of flavors, especially spray-dried flavors. They are classified by their cold water solubility and color. Dextrins that retain large amounts of linear chains or long chain fragments form strong gels.

Incomplete hydrolysis of cooked, that is, pasted, starch dispersions with either an acid or an enzyme produces mixtures of maltooligosaccharides,* which are referred to industrially as maltodextrins. Maltodextrins are usually described by their dextrose equivalency (DE). The DE is related to the DP through the following equation:

$$\mathrm{DE} = 100/\mathrm{DP}$$

where both DE and DP are average values for populations of molecules. Therefore, the DE of a product of hydrolysis is its reducing power as a percentage of the reducing power of pure D-glucose (dextrose); thus, DE is inversely related to average molecular weight. Maltodextrins are defined as products with DE values that are measurable, but <20, that is, their average DPs are >5. Maltodextrins of lowest DE, that is, highest average molecular weight, are nonhygroscopic, while those of highest DE tend to absorb moisture. Maltodextrins are bland with virtually no sweetness and are excellent for contributing body or bulk to food systems. Hydrolysis to DE values of 20–60 gives mixtures of molecules that, when dried, are called corn syrup solids. Corn syrup solids dissolve rapidly and are mildly sweet.

* Oligosaccharides from starch are known as maltooligosaccharides.

Continued hydrolysis of starch produces a mixture of D-glucose, maltose, and other maltooligosaccharides. Syrups with these components in various ratios are produced in enormous quantities. One of the most common has a DE of 42. These syrups are stable because crystallization does not occur easily in such complex mixtures. They are sold in concentrations of high osmolality (about 70% solids), high enough so that ordinary organisms cannot grow in them. An example is waffle and pancake syrup, which is colored with caramel coloring and flavored with maple flavoring.

Three to four enzymes are used for the industrial hydrolysis of starch to D-glucose. α-Amylase is an endo-enzyme that cleaves both amylose and amylopectin molecules internally, producing oligosaccharides. The larger oligosaccharides may be singly, doubly, or triply branched via (1→6) linkages, since α-amylase acts only on the (1→4) linkages of starch. α-Amylase does not attack double-helical starch polymer segments or polymer segments complexed with a polar lipid (stabilized single-helical segments).

Glucoamylase (amyloglucosidase) is used commercially, in combination with an α-amylase, for producing D-glucose (dextrose) syrups and crystalline D-glucose. The enzyme acts upon fully gelatinized starch as an exo-enzyme, sequentially releasing single D-glucosyl units from the nonreducing ends of amylose and amylopectin molecules, even those joined through (1→6) bonds. Consequently, the enzyme can completely hydrolyze starch to D-glucose, but is always used on starch that has already been depolymerized with α-amylase to generate fragments and, thus, more nonreducing ends.

β-Amylase releases the disaccharide maltose sequentially from the nonreducing ends of starch polymer chains. When amylopectin is the substrate, it attacks the nonreducing ends, sequentially releasing maltose, but it cannot cleave the (1→6) linkages at branch points; so it leaves a pruned amylopectin residue termed a limit dextrin, specifically a β-limit dextrin.

There are several debranching enzymes that specifically catalyze hydrolysis of (1→6) linkages in amylopectin, producing numerous linear, but low-molecular-weight molecules. One such enzyme is isoamylase; another is pullulanase.

Cyclodextrin glucanotransferase is a unique *Bacillus* enzyme that forms rings of (1→4)-linked α-D-glucopyranosyl units from starch polymers called cyclodextrins (Section 3.2.4). Glucose syrup, often called corn syrup in the United States, is the major source of D-glucose and D-fructose. To make a syrup, a slurry of starch in water is mixed with a thermally stable α-amylase and put through a special cooker where rapid gelatinization and enzyme-catalyzed hydrolysis (liquefaction) takes place. After cooling to 55–60°C (130–140 F), hydrolysis is continued with glucoamylase, whereupon the syrup is clarified, concentrated, carbon-refined, and ion-exchanged. If the syrup is properly refined and combined with seed crystals, crystalline D-glucose (dextrose) can be obtained.

For production of D-fructose, a D-glucose solution is passed through a column containing bound (immobilized) glucose isomerase. The enzyme catalyzes the isomerization of D-glucose to D-fructose (see Figure 3.5), forming an equilibrium mixture of approximately 58% D-glucose and 42% D-fructose. Higher concentrations of D-fructose are usually desired. (The HFS most often used as a soft drink sweetener contains approximately 55% D-fructose.) To make a syrup with a concentration of D-fructose greater than 42%, the isomerized syrup is passed through a bed of cation-exchange resin in the calcium salt form. The resin binds D-fructose that can be recovered and added to the normal syrup to produce a syrup enriched in D-fructose.

3.3.6.10 Modified Food Starches [5,66,68]

Food processors generally prefer starches with better behavioral characteristics than provided by native starches. Native starches produce weak-bodied, cohesive, rubbery pastes when cooked and undesirable gels when the pastes are cooled. Modification is done to improve the characteristics of the pastes and gels. Some modifications are done so that resultant pastes can withstand the conditions of heat, shear, and acid associated with particular processing conditions; others are done to introduce specific functionalities. Modified food starches are functional, useful, and abundant food macroingredients and additives.

Modifications can be chemical or physical. Chemical modifications make crosslinked, stabilized, oxidized, and depolymerized (acid-modified, thin-boiling; Section 3.3.6.9) products. Physical modifications make pregelatinized (Section 3.3.6.11) and cold-water-swelling (Section 3.3.6.12) products. Chemical modifications have the greatest effects on functionalities, and the majority of modified food starch products have been derivatized with reagents that react with hydroxyl groups to form ethers or esters. Modifications can be single modifications, but modified starches often are prepared by combinations of two, three, and sometimes four processes.

Chemical reactions currently both allowed and used to produce modified food starches in the United States are as follows: esterification with acetic anhydride, succinic anhydride, the mixed anhydride of acetic and adipic acids, 1-octenylsuccinic anhydride, phosphoryl chloride, sodium trimetaphosphate, sodium tripolyphosphate, and monosodium orthophosphate; etherification with propylene oxide; acid modification with hydrochloric and sulfuric acids; bleaching with hydrogen peroxide, peracetic acid, potassium permanganate, and sodium hypochlorite; oxidation with sodium hypochlorite; and various combinations of these reactions.

Approved and used esterified and etherified modified food starches include the following:

Stabilized starches

- Hydroxypropyl starches (starch ether)
- Starch acetates (starch ester)
- Starch octenylsuccinates (monostarch ester)
- Monostarch phosphate (ester)

Crosslinked starches

- Distarch phosphate
- Distarch adipate

Crosslinked and stabilized starches

- Hydroxypropylated distarch phosphate
- Phosphorylated distarch phosphate
- Acetylated distarch phosphate
- Acetylated distarch adipate

Crosslinked starches have higher gelatinization and pasting temperatures, increased resistance to shear, and increased stability to low pH conditions and produce pastes with greater viscosities and stability as compared to the base starch.

Stabilized products have lower gelatinization and pasting temperatures, are easier to redisperse when pregelatinized, and produce pastes and gels with a reduced tendency for retrogradation, that is, greater stability, improved freeze–thaw stability, and greater clarity as compared to the base starch.

Hypochlorite-oxidized products are whiter, have lower gelatinization and pasting temperatures, produce a lower maximum paste viscosity, and result in softer, clearer gels as compared to the unmodified starch.

Starches that have been both crosslinked and stabilized generally have lowered gelatinization and pasting temperatures, produce pastes with greater viscosity, and demonstrate the other attributes of crosslinking and stabilization as compared to the base starch.

Thinned, that is, very slightly depolymerized, products have lower gelatinization and pasting temperatures and produce pastes with less viscosity as compared to the base starch.

Any starch (corn, waxy maize, potato, tapioca/cassava, wheat, rice, etc.) can be modified, but modification is practiced significantly only on normal corn, waxy maize, and potato starches and to a much lesser extent on tapioca and wheat starches. Modified waxy maize starches are especially popular in the U.S. food industry. Pastes of unmodified common corn starch will gel, and the gels will

generally be cohesive, rubbery, and prone to syneresis (i.e., to weep or exude moisture). Pastes of waxy maize starch show little tendency to gel at room temperature, which is why waxy maize starch is generally preferred as the base starch for food starches, but pastes of waxy maize starch will become cloudy and chunky and exhibit syneresis when stored under refrigerator or freezing conditions; so even waxy maize starch is usually modified to increase the stability of its pastes. The most common and useful derivative employed for starch stabilization is the hydroxypropyl ether (see later).

Specific property improvements that can be obtained by proper combinations of modifications are reduction in the energy required to cook (improved gelatinization and pasting), modification of cooking behaviors, increased solubility, either increased or decreased paste viscosity, increased freeze–thaw stability of pastes, enhancement of paste clarity, increased paste sheen, reduction or enhancement of gel formation and gel strength, reduction of gel syneresis, improvement of interaction with other substances, improvement in stabilizing properties, enhancement of film formation, improvement in water resistance of films, reduction in paste cohesiveness, and improvement of stability to acid, heat, and shear.

Starch, like all carbohydrates, can undergo reactions at its various hydroxyl groups. In modified food starches, only a very few of the hydroxyl groups are modified. Normally, ester or ether groups are attached at very low degrees of substitution (DS) values.* DS values are often <0.1 and generally in the range 0.002–0.2, depending on the modification. Thus, there is, on average, one substituent group on every 500-5 D-glucopyranosyl units, respectively. Small levels of derivatization change the properties of starches dramatically and greatly extend their usefulness.

Starch products that are esterified or etherified with monofunctional reagents resist interchain associations, which reduces the tendency of the starch paste to gel, and the tendency for precipitation to occur. Hence, this modification is often called stabilization and the products are called stabilized starches (see below). Use of bifunctional reagents produces crosslinked starches. Modified food starches are often both crosslinked and stabilized.

Acetylation of starch to the maximum allowed in foods (DS = 0.09) lowers the gelatinization temperature, improves paste clarity, provides stability to retrogradation, and provides some freeze–thaw stability (but generally not as effectively as hydroxypropylation). Starch phosphate monoesters (Figure 3.44) are made by treating starch with sodium tripolyphosphate or monosodium orthophosphate. They can be used to make pastes that are clear and stable and have freeze–thaw stability. Monostarch phosphates have a long, cohesive texture. Paste viscosity is generally high and can be controlled by varying the concentration of reagent, time of reaction, temperature, and pH. Phosphate esterification lowers the gelatinization temperature. In the United States, the maximum allowable DS with phosphate groups is 0.002.

Preparation of an alkenylsuccinate ester of starch attaches a hydrophobic, hydrocarbon chain to its polymer molecules (Figure 3.45). Even at very low DS, starch 1-octenylsuccinate molecules concentrate at the interface of an oil-in-water emulsion because of the hydrophobicity of the alkenyl group. This characteristic makes them useful as emulsion stabilizers. Starch 1-octenylsuccinate products can be used in a variety of food applications where emulsion stability is needed, such as in flavored beverages. The presence of the aliphatic chain tends to give the starch derivative a sensory perception of fattiness, so it is possible to use the derivative as a partial replacement for fat in certain foods. Higher-DS products are nonwetting and are used as release agents for dusting on dough sheets and as processing aids.

Hydroxypropylation is the most often used reaction to prepare a stabilized starch product. Hydroxypropylstarch (starch$-O-CH_2-CHOH-CH_3$) is prepared by reacting starch with propylene oxide to produce a low level of etherification (DS 0.02–0.2, 0.2 being the maximum

* The degree of substitution (DS) is defined as the average number of esterified or etherified hydroxyl groups per monosaccharide unit. Both branched and unbranched polysaccharides composed of neutral hexopyranosyl units have an average of three hydroxyl groups per monomeric unit. Therefore, the maximum DS for a starch and cellulose is 3.0, although the maximum possible is not allowed in products used as food ingredients.

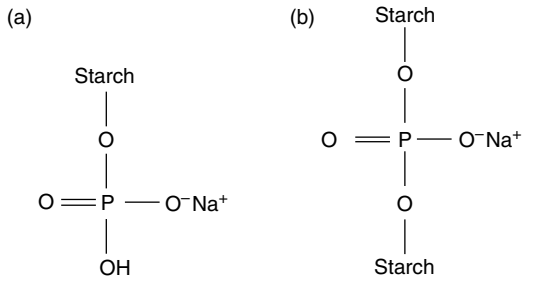

FIGURE 3.44 Structures of starch monoester phosphate (a) and diester phosphate (b). The diester joins two molecules together, resulting in crosslinked starch granules.

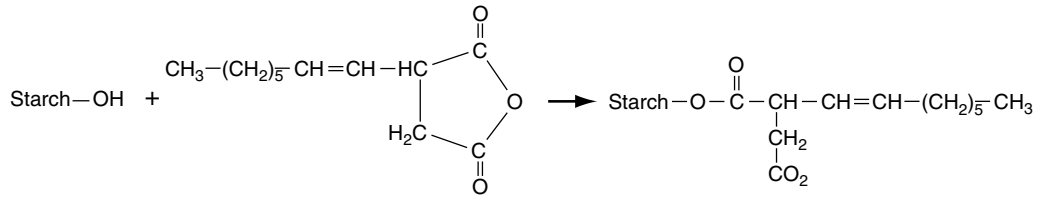

FIGURE 3.45 Preparation of the 2-(1-octenyl)succinyl ester of starch.

allowed). Hydroxypropylstarch has properties similar to those of starch acetate, because it similarly has "bumps" along the starch polymer chains that prevent the interchain associations that lead to retrogradation. Hydroxypropylation reduces the gelatinization temperature. Hydroxypropylstarches form clear pastes that do not retrograde and withstand freezing and thawing. They are used as thickeners and extenders. To improve viscosity, particularly under acidic conditions, acetylated and hydroxypropylated starches are often also crosslinked with phosphate groups.

Monostarch phosphates (sodium phosphate monoesters of starch) are prepared by impregnating and reacting starch granules with solutions of sodium tripolyphosphate or monosodium orthophosphate. Monostarch phosphates produce stable pastes that are clear and have a long, cohesive texture. Pastes viscosity can be controlled by varying the concentrations of phosphate salt, time of reaction, temperature, and pH. Increasing substitution lowers the gelatinization temperature; products undergo cold-water swelling at DS 0.07. Corn starch phosphates of DS 0.01–0.03 produce pastes with hot viscosity, clarity, stability, and texture more like those of potato starch. Starch phosphates are good emulsion stabilizers and produce pastes with improved freeze–thaw stability.

The majority of modified food starch is crosslinked. Crosslinking occurs when starch granules are reacted with bifunctional reagents that react with hydroxyl groups on two different molecules within the granule. Crosslinking is accomplished most often by producing distarch phosphate esters (Figure 3.44). Starch is either reacted with phosphoryl chloride ($POCl_3$) in an alkaline slurry or reacted with sodium trimetaphosphate—$POCl_3$ being the reagent most often used for crosslinking. The linking together of starch chains with phosphate diester or other crosslinks reinforces the granule and reduces both the rate and the degree of granule swelling and subsequent disintegration. Thus, granules exhibit reduced sensitivity to processing conditions (high temperature; extended cooking times; low pH; high shear during mixing, milling, homogenization, and/or pumping). Cooked pastes of crosslinked starches are more viscous,* heavier bodied, shorter textured, and less likely to break down during extended cooking or during exposure to low pH and/or severe agitation than

* Note in Figure 3.42 that maximum viscosity is reached when the system contains highly swollen granules. Crosslinked granules are less prone to disintegrate as shear is applied. Thus, there is less loss of viscosity after the peak is reached.

are pastes of the native starches from which they are prepared. Only a small amount of crosslinking is required to produce a noticeable effect; and with lower levels of crosslinking, granules swell in inverse proportion to DS. As crosslinking is increased, the granules become more and more tolerant to physical conditions and acidity, but less and less dispersible by cooking. Energy requirements to reach maximum swelling and viscosity are increased. For example, treatment of a starch with only 0.0025% of sodium trimetaphosphate greatly reduces both the rate and the degree of granule swelling, greatly increases paste stability, and changes dramatically the pasting/paste viscosity profile and textural characteristics of its paste. Treatment with 0.08% of trimetaphosphate produces a product in which granule swelling is restricted to the point that a peak viscosity is never reached during the hot holding period. As the degree of crosslinking increases, the starch also becomes more acid stable. Though hydrolysis of glycosidic bonds occurs during heating in aqueous acid, chains tied to each other through phosphate crosslinks continue to provide large molecules and an elevated viscosity. The only other crosslink permitted in a food starch is the distarch ester of adipic acid.

Most crosslinked food starches contain less than one crosslink per 1000 α-D-glucopyranosyl units. Trends toward continuous cooking require increased shear-resistance and stability to hot surfaces. Storage-stable thickening is also provided by crosslinked starches. In retort sterilization of canned foods, crosslinked starches, because of their reduced rate of gelatinization and swelling, maintain a low initial viscosity long enough to facilitate the rapid heat transfer and temperature rise that is needed to provide uniform sterilization before granule swelling brings about the desired viscosity, texture, and suspending characteristics. Crosslinked starches are used in canned soups, gravies, and puddings and in batter mixes. Crosslinking of waxy maize starch gives the clear paste sufficient rigidity so that, when used in pie fillings, the cut sections hold their shape.

Depolymerization, viscosity reduction, and decreased pasting temperature can be achieved by oxidation with sodium hypochlorite (chlorine in an alkaline solution). Oxidation also reduces association of amylose molecules, that is, results in some stabilization via introduction of small amounts of carboxylate and carbonyl groups. Oxidized starches produce less viscosity and softer gels (as compared with the base starch) and are used when these properties are needed. They are also used to improve adhesion of starch batters to fish and meat and in breading. Mild treatment with sodium hypochlorite, hydrogen peroxide, or potassium permanganate simply bleaches the starch and reduces the count of viable microbes.

So-called thin-boiling starches are prepared by treating a suspension of a native or derivatized starch with dilute mineral acid at a temperature below the gelatinization temperature. When a product that gives the desired paste viscosity is reached, the acid is neutralized, and the product is recovered washed, and dried. Even though only a few glycosidic bonds are hydrolyzed, granules disintegrate more easily and after only a small degree of swelling. Acid-modified starches form gels with improved clarity and increased strength, even though their pastes are less viscous. Thin-boiling starches are used as film formers and adhesives in products such as pan-coated nuts and candies and whenever a strong gel is desired, for example, in gum candies such as jelly beans, jujubes, orange slices, and spearmint leaves and in processed cheese loaves. To prepare especially strong and fast-setting gels, a high-amylose corn starch is used as the base starch.

Modified food starches are tailor-made for specific applications. Properties that can be controlled by combinations of crosslinking, stabilization, and thinning of corn, waxy maize, potato, wheat, and other starches include, but are not limited to, the following: adhesion, clarity of solutions/pastes, color, emulsion stabilization ability, film forming ability, flavor release, hydration rate, moisture holding capacity, stability to acids, stability to heat and cold, stability to shear, temperature required to cook, and viscosity (hot paste and cold paste). Some characteristics imparted to the food product include, but are not limited to, the following: mouthfeel, reduction of oil migration, texture, sheen, stability, and tackiness.

Starches that are both crosslinked and stabilized are used in canned, frozen, baked, and dry foods. In baby foods and fruit pie fillings in cans and jars, they provide long shelf life. They also allow frozen fruit pies, pot pies, and gravies to remain stable under long-term storage.

3.3.6.11 Cold-Water-Soluble (Pregelatinized or Instant) Starch

Starch that has been pasted/cooked and dried without excessive retrogradation can be partially redissolved in cold water. Such starch is called pregelatinized or instant starch. It has been gelatinized, but it has also been pasted, that is, many granules have been destroyed; so it should more properly be called precooked or prepasted starch. There are two basic approaches to making pregelatinized products. In one, a starch–water slurry is introduced into the nip between two nearly touching and counterrotating, steam-heated rolls or applied to the top of a single rotating, steam-heated roll. In either case, the starch slurry is gelatinized and pasted almost instantaneously, and the paste that coats the rolls dries rapidly. The dry film is scraped from the roll and ground to a powder. The resulting products are cold-water soluble and will produce viscous dispersions when stirred into room-temperature water, although some heating is often required to achieve maximum viscosity. The second method of preparation uses extruders. In this process the heat and shear in an extruder gelatinizes and destroys the moistened starch granules. The puffed, crispy, glassy extrudate is ground to a powder.

Both chemically modified and unmodified starches can be used to make pregelatinized starches. If chemically modified starches (Section 3.3.6.10) are used, properties introduced by the modification(s) carry through to the pregelatinized products; thus, paste properties such as stability to freeze–thaw cycling can also be characteristics of pregelatinized starches. Pregelatinized, slightly crosslinked starch is useful in instant soup, pizza topping, and extruded snacks and breakfast cereals.

The advantage of pregelatinized starches is that they can be used without cooking. Like a water-soluble gum, finely ground pregelatinized starch forms small gel particles when added to water, but when properly dispersed and dissolved, gives solutions of high viscosity. More coarsely ground products disperse more easily and produce dispersions of lower viscosity and with a graininess or pulpiness that is desirable in some products. Many pregelatinized starches are used in dry mixes, such as instant pudding mixes. They disperse readily with high-shear stirring or when mixed with sugar or other dry ingredients.

3.3.6.12 Cold-Water-Swelling Starch

Granular starch that swells extensively in cold water is made by heating common corn starch in 75–90% ethanol or by a special spray-drying process. This product is also categorized as a pregelatinized or instant starch by some. The difference between it and conventional pregelatinized starch is that, while the crystalline order and birefringence of the granules have been destroyed by the treatment, the granules are intact. Therefore, when added to water, they swell as if they were being cooked. The dispersion made by incorporating cold-water-swelling starch into sugar solutions or glucose syrups by rapid stirring can be poured into molds, where it sets to a rigid gel that can be sliced. The result is a gum candy. Cold-water-swelling starch is also useful in making desserts and in muffin batters containing particles, such as blueberries, that otherwise would settle to the bottom before the batter is thickened by heating during baking.

3.3.7 Cellulose: Forms and Derivatives [71]

Cellulose is a high-molecular-weight, linear, insoluble homopolymer of repeating β-D-glucopyranosyl units joined by (1→4) glycosidic linkages (Figure 3.46). The axial → equatorial (1→4) linkages joining the α-D-glucopyranosyl units of starch polymer molecules produce a coiled structure (an α-helix). In contrast, the equatorial → equatorial (1→4) linkages joining the β-D-glucopyranosyl units of cellulose molecules give them a flat, ribbon-like structure in which each glucopyranosyl unit in the chain is turned upside down as compared to the units preceding and following it. Because of their flat and linear nature, cellulose molecules can associate with each

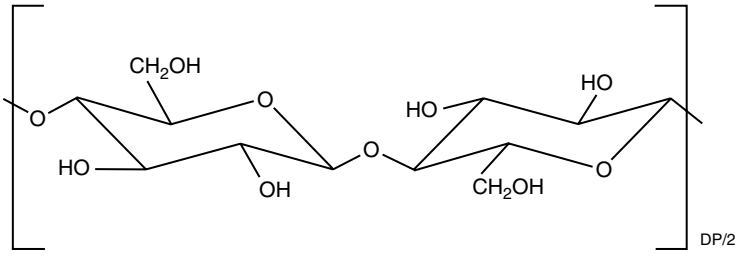

FIGURE 3.46 Cellulose (repeating unit).

other via hydrogen bonding over extended regions, forming polycrystalline, fibrous bundles. Crystalline regions are separated by, and connected to, amorphous regions. Cellulose is insoluble in water because, in order for it to dissolve, most of its very many hydrogen bonds would have to be released at once. Cellulose can, however, through derivatization, be converted into water-soluble gums.

Cellulose and its modified forms serve as dietary fiber because they are undigested and do not contribute significant nourishment or calories as they pass through the human digestive system. Dietary fiber is important to human nutrition (see Section 3.4).

A purified cellulose powder is available as a food ingredient. High-quality cellulose can be obtained from wood through pulping and subsequent purification. Chemical purity is not required for food use because cellulosic cell walls are components of all fruits and vegetables. The powdered cellulose used in foods has negligible flavor, color, and microbial contamination. Powdered cellulose is most often added to bread to provide noncaloric bulk. Reduced-calorie baked goods made with powdered cellulose not only have an increased content of dietary fiber, but also stay moist and fresh longer.

3.3.7.1 Microcrystalline Cellulose [58]

A purified, insoluble cellulose termed microcrystalline cellulose (MCC) is made by partial hydrolysis of purified wood pulp cellulose, with hydrolysis taking place in the amorphous regions, followed by separation of the released microcrystals. Cellulose molecules are fairly rigid, completely linear chains of about 3000 β-D-glucopyranosyl units and associate easily in long junction zones. However, the long and unwieldy chains do not align over their entire lengths. The end of the crystalline region is simply the divergence of cellulose chains away from order into a more random arrangement, forming the amorphous region. When purified wood pulp is hydrolyzed with acid, the acid penetrates the lower-density, hydrated amorphous regions where the polymer chains have greater freedom of movement and effects hydrolytic cleavage of chains in these regions, releasing individual, fringed crystallites.

Two types of MCC are produced, both of which are stable to both heat and acids. Powdered MCC is a spray-dried product. Spray-drying produces porous aggregates of microcrystals. Powdered MCC is used as a flavor carrier and as an anticaking agent for shredded cheese. The second type, colloidal MCC, is water dispersible and has functional properties similar to those of water-soluble gums. To make colloidal MCC, considerable mechanical energy is applied after hydrolysis to tear apart the weakened microfibrils and provide a major proportion of colloidal-sized aggregates (<0.2 μm in diameter). To prevent rebonding of the aggregates during drying, sodium CMC (Section 3.3.7.2), xanthan (Section 3.3.9), or sodium alginate (Section 3.3.11) is added. The anionic gum aids in redispersion and acts as a barrier to reassociation by giving the particles a stabilizing negative charge.

The major functions of colloidal MCC are to stabilize foams and emulsions, especially during high-temperature processing; to form gels with salve-like textures (MCC does not dissolve, nor does it form intermolecular junction zones; rather it forms a network of hydrated microcrystals);

to stabilize pectin and starch gels to heat; to improve adhesion; to replace fat and oil in products like salad dressings and ice cream; and to control ice crystal growth. MCC stabilizes emulsions and foams by adsorbing at interfaces and strengthening interfacial films. It is a common ingredient of reduced fat ice cream and other frozen dessert products.

3.3.7.2 Carboxymethylcelluloses [14,33]

Carboxymethylcellulose (Table 3.5) is widely and extensively used as a food gum. Treatment of purified wood pulp with 18% sodium hydroxide solution produces alkali cellulose. When alkali cellulose is reacted with the sodium salt of chloroacetic acid, the sodium salt of the carboxymethyl ether (cellulose—O—CH_2—$CO_2^-Na^+$) is formed. Most commercial CMC products have a DS (see Section 3.3.6.10) in the range 0.4–0.8. The most widely sold type for use as a food ingredient has a DS of 0.7.

Since CMC consists of long, fairly rigid molecules that bear a negative charge due to numerous ionized carboxyl groups, electrostatic repulsion causes its molecules in solution to be extended. Also, adjacent chains repel each other. Consequently, CMC solutions tend to be both highly viscous and stable. CMC is available in a wide range of viscosity grades. CMC stabilizes protein dispersions, especially near the isoelectric pH value of the protein.

3.3.7.3 Methylcelluloses and Hydroxypropylmethylcelluloses [24,25]

To make methylcellulose (MC) products (Table 3.5), alkali cellulose is treated with methyl chloride to introduce methyl ether groups (cellulose—O—CH_3). Many members of this family of gums also contain hydroxypropyl ether groups (cellulose—O—CH_2—CHOH—CH_3). Hydroxypropylmethylcelluloses (HPMCs) are made by reacting alkali cellulose with both propylene oxide and methyl chloride. The DS with methyl ether groups of commercial MCs ranges from 1.1 to 2.2. The moles of substitution (MS)* with hydroxypropyl ether groups in commercial HPMCs range from 0.02 to 0.3. (Both the MC and HPMC members of this gum family are generally referred to simply as MCs.) Both products are cold-water soluble because the methyl and hydroxypropyl ether group protrusions along the chains prevent the intermolecular association characteristic of cellulose.

While a few added ether groups spread along the chains and enhance water solubility, they also decrease chain hydration by replacing water-binding hydroxyl groups with less polar ether groups, giving members of this family unique characteristics. The ether groups restrict solvation of the chains to the point that they are on the borderline of water solubility. When an aqueous solution is heated, the water molecules hydrating the polymer dissociate from the chain and hydration is decreased sufficiently so that intermolecular associations increase (probably via van der Waals interactions) and gelation occurs. Lowering the temperature of the gel allows the molecules to rehydrate and redissolve, so the gelation is reversible.

Because of the ether groups, the gum chains are somewhat surface active and absorb at interfaces. This helps stabilize emulsions and foams. MCs also can be used to reduce the amount of fat in food products through two mechanisms: (1) they provide fat-like properties so that the fat content of a product can be reduced and (2) they reduce adsorption of fat in products being fried, for the gel structure produced by thermogelation provides a barrier to oil, holds moisture, and acts as a binder.

* The moles of substitution or molar substitution (MS) value indicates the average number of moles of substituent attached to a glycosyl unit of a polysaccharide. Because reaction of a hydroxyl group with propylene oxide creates a new hydroxyl group with which propylene oxide can react further, poly(propylene oxide) chains, each terminated with a free hydroxyl group, can form. Because more than three moles of propylene oxide can react with a single hexopyranosyl unit, MS rather than DS is used.

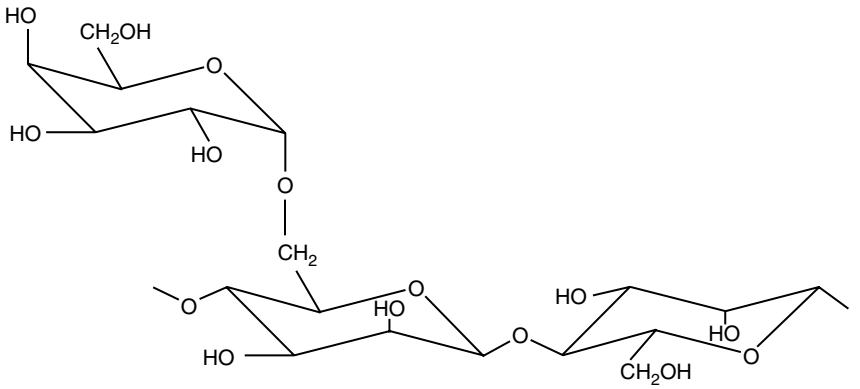

FIGURE 3.47 A representative segment of a galactomannan molecule.

3.3.8 Guar and Locust Bean Gums [27,28,38]

Guar and LBGs are important thickening polysaccharides (Table 3.5). Guar gum produces the highest viscosity of any natural, commercial gum. Both gums are the ground endosperm of seeds. The main component of both endosperms is a galactomannan. Galactomannans consist of a main chain of β-D-mannopyranosyl units joined by (1→4) bonds with single-unit α-D-galactopyranosyl branches attached at O-6 (Figure 3.47). The specific polysaccharide that makes up most of guar gum is guaran. In guaran, about one-half of the D-mannopyranosyl main chain units contain an α-D-galactopyranosyl unit.

The galactomannan of LBG (also called carob gum) has fewer branch units than does guaran and its structure is more irregular, with long stretches of about 80 underivatized D-mannosyl units alternating with sections of about 50 units in which most of the main chain units have an α-D-galactopyranosyl group glycosidically connected to their O-6 positions.

Because of the difference in structures, guar gum and LBG have different physical properties, even though both are galactomannans and are composed of long, rather rigid chains that provide high solution viscosity. Because guaran has its galactosyl units fairly evenly placed along the chain, there are few locations on the chains that are suitable for formation of junction zones. However, LBG with its long "naked chain" sections, can form junction zones. LBG molecules interact with xanthan (Figure 3.48; Section 3.3.9) and carrageenan (Section 3.3.10) helices, forming junction zones and rigid gels.

Guar gum provides economical thickening to numerous food products. It is frequently used in combination with other food gums, for example, in ice cream, where it is often used in combination with CMC (Section 3.3.7.2), carrageenan (Section 3.3.10), and LBG.

Typical products in which LBG is found are the same as those for guar gum. About 85% of LBG is used in dairy and frozen dessert products. It is rarely used alone; rather it is used in combination with other gums such as CMC, carrageenan, xanthan, and guar gum. It is used in combination with κ-carrageenan and xanthan to take advantage of the synergistic gel-forming phenomenon. A typical use level is 0.05–0.25%.

3.3.9 Xanthan [32,47]

Xanthomonas campestris, a bacterium commonly found on leaves of plants of the cabbage family, produces a polysaccharide, termed xanthan, that is produced in large fermentation vats and is widely used as a food gum. The polysaccharide is known commercially as xanthan gum (Table 3.5).

Xanthan has a backbone chain identical to that of cellulose (Figure 3.48; compare with Figure 3.46). In the xanthan molecule, every other β-D-glucopyranosyl unit in the cellulose backbone

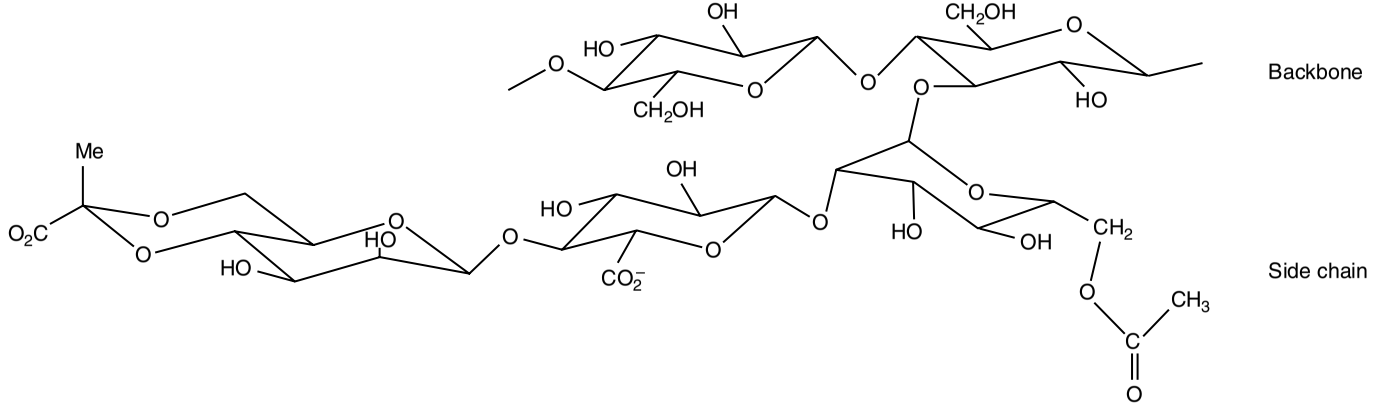

FIGURE 3.48 Structure of the pentasaccharide repeating unit of xanthan. Note the 4,6-*O*-pyruvyl-D-mannopyranosyl nonreducing end-unit of the trisaccharide side chain. Normally, about one-half of the side chains are pyruvylated.

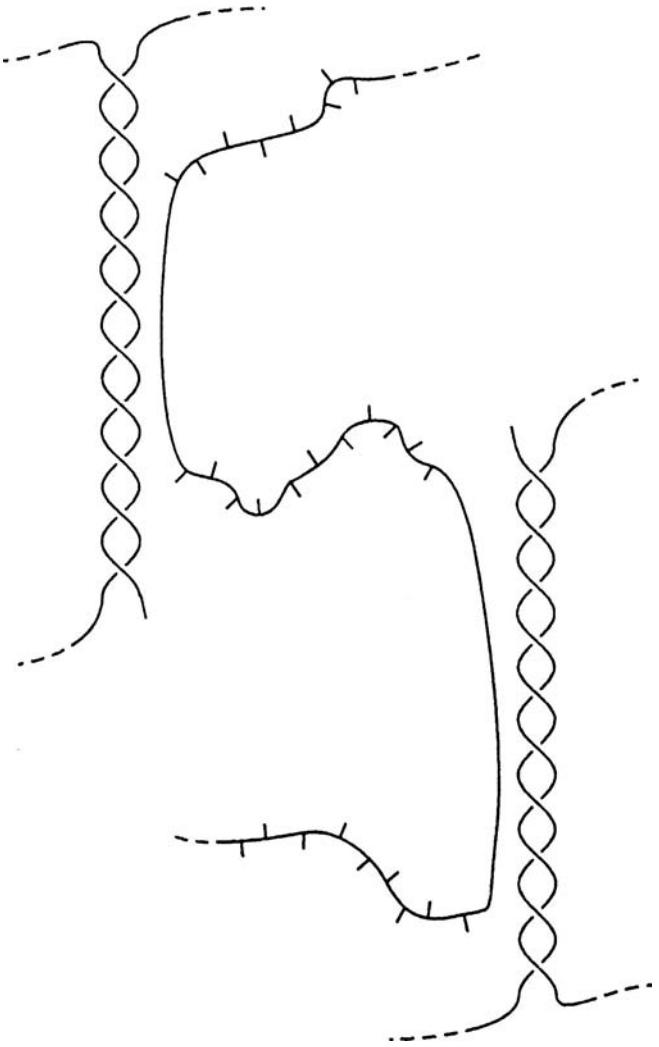

FIGURE 3.49 Representation of the hypothesized interaction of a locust bean gum molecule with double-helical portions of xanthan or carrageenan molecules to form a three-dimensional network and a gel.

has attached, at the O-3 position, a β-D-mannopyranosyl-(1→4)-β-D-glucuronopyranosyl-(1→2)-6-*O*−acetyl-β-D-mannopyranosyl trisaccharide unit.* About half of the terminal β-D-mannopyranosyl units have pyruvic acid attached as a 4,6-cyclic acetal. The trisaccharide side chains interact with the main chain and make the molecule rather stiff. The molecular weight is probably in the order of 2×10^6, although much larger values, presumably due to aggregation, have been reported.

Xanthan interacts with guar gum synergistically to produce an increase in solution viscosity. The interaction with LBG produces a heat-reversible gel (Figure 3.49).

Xanthan is widely used as a food gum because of the following important characteristics: it is soluble in both hot and cold water; it produces high solution viscosity at low concentrations; there is no discernible change in solution viscosity in the temperature range from 0 to 100°C, which makes it unique among food gums; it is both soluble and stable in acidic systems; it has excellent

* Bacterial heteroglycans, unlike plant heteroglycans, have regular, repeating unit structures.

compatibility with salt; it forms gels when used in combination with LBG; it is a remarkable stabilizer of suspensions and emulsions; and it imparts stability to products exposed to freezing and thawing. The unusual and very useful properties of xanthan undoubtedly result from the structural rigidity and extended nature of its molecules, which in turn result from its linear, cellulosic backbone that is stiffened and shielded by the anionic trisaccharide side chains.

Xanthan is ideal for stabilizing aqueous dispersions, suspensions, and emulsions. The fact that the viscosity of its solutions changes very little with temperature, that is, its solutions do not thicken upon cooling, make it irreplaceable for thickening and stabilizing such products as pourable salad dressings and chocolate syrup, which need to pour as easily when taken from the refrigerator as they do at room temperature, and gravies, which should neither thicken appreciably as they cool nor thin too much when hot. In regular, pourable salad dressings, it serves both as a thickener and as a stabilizer for both the suspension of particulate materials and the oil-in-water emulsion. It is also used as a thickener and suspending agent in no-oil (reduced-calorie) dressings. In both oil-containing and no-oil salad dressings, xanthan is almost always used in combination with propylene glycol alginate (PGA) (Section 3.3.11). PGA decreases the viscosity of the xanthan-containing system and reduces its pseudoplasticity. Together they give the desired pourability associated with the pseudoplastic xanthan and the creaminess sensation associated with a nonpseudoplastic solution.

3.3.10 Carrageenans, Agar, and Furcellaran [22,57]

The term carrageenan denotes a group or family of sulfated galactans extracted from red seaweeds with dilute alkaline solutions; the sodium salt of a carrageenan is normally produced. Carrageenans are mixtures of several related sulfated galactans (Table 3.5). Carrageenans are linear chains of D-galactopyranosyl units joined with alternating (1→3)-α-D- and (1→4)-β-D-glycosidic linkages, with most sugar units having one or two sulfate half-ester groups esterified to the hydroxyl groups at carbon atoms C-2 and/or C-6. This gives a sulfate content ranging from 15 to 40%. Units often contain a 3,6-anhydro ring. The principal structures are termed kappa (κ), iota (ι), and lambda (λ) carrageenans (Figure 3.50). The disaccharide units shown in Figure 3.50 represent the predominate building block of each type, but are not repeating unit structures. Carrageenans, as extracted, are mixtures of nonhomogeneous polysaccharides. Carrageenan products, of which there may be more than 100 from a single supplier for different specific applications, contain different proportions of the three main behavioral types (kappa, iota, and lambda) produced by starting with mixtures of red seaweed species. Other substances, such as potassium ions and sugar (for standardization), may be added to the obtained powder.

Carrageenan products dissolve in water to form highly viscous solutions. The viscosity is quite stable over a wide range of pH values because the sulfate half-ester groups are always ionized, even under strongly acidic conditions, giving the molecules a negative charge. However, carrageenans undergo depolymerization in hot acidic solutions, so these conditions are to be avoided when using a carrageenan product.

Segments of molecules of κ- and ι-type carrageenans exist as double helices of parallel chains. In the presence of potassium or calcium ions, thermoreversible gels form upon cooling a hot solution containing double-helical segments. Gelation can occur in water at concentrations as low as 0.5%. When κ-type carrageenan solutions are cooled in the presence of potassium ions, a stiff, brittle gel results. Calcium ions are less effective in causing gelation. Potassium and calcium ions together produce a high gel strength. Gels made with κ-type carrageenans are the strongest of the carrageenan gels. These gels tend to synerese as junction zones within the structure grow in length. The presence of other gums retards syneresis.

ι-Type carrageenans are a little more soluble than are the κ-types, but again, only the sodium salt form is soluble in cold water. ι-Types gel best with calcium ions. The resulting gel is soft and resilient, has good freeze–thaw stability, and does not synerese, presumably because ι-type carrageenans are more hydrophilic and form fewer junction zones than do κ-type carrageenans.

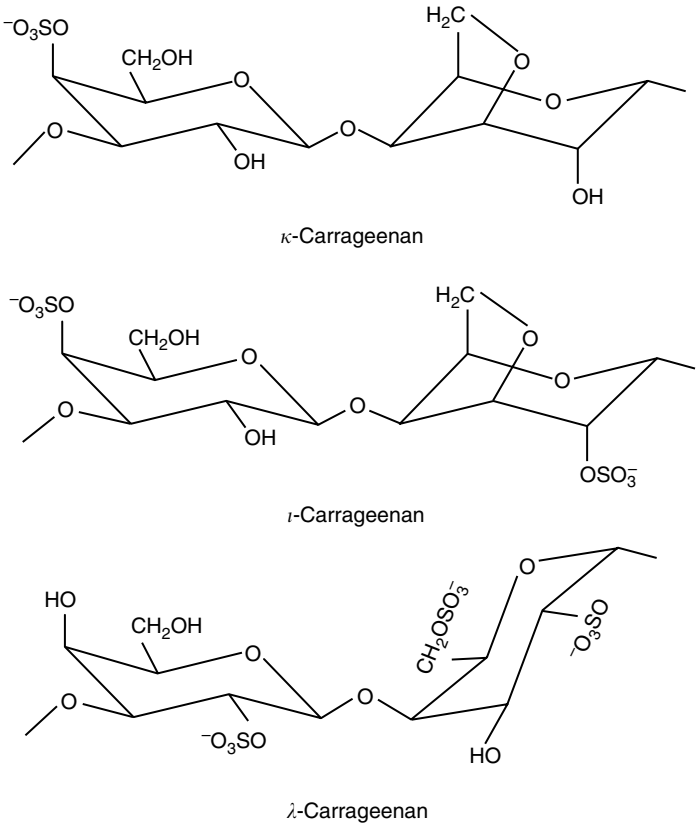

FIGURE 3.50 Idealized unit structures of κ-, ι-, and λ-type carrageenans.

During cooling of solutions of κ- or ι-type carrageenans, gelation occurs because the linear molecules are unable to form continuous double helices due to the presence of structural irregularities. The linear helical portions then associate to form a three-dimensional gel in the presence of the appropriate cation (Figure 3.51). All salts of λ-type carrageenans are soluble and nongelling.

Under conditions in which double-helical segments are present, carrageenan molecules, particularly those of the κ-type, form junction zones with the naked segments of LBG to produce rigid, brittle, syneresing gels. This gelation occurs at a concentration one-third that needed to form a pure κ-type carrageenan gel.

Carrageenans are most often used because of their ability to form gels with milk and water. Mixtures of carrageenan types are used to provide a wide range of products that are standardized with various amounts of sucrose, glucose (dextrose), buffer salts, or gelling aids, such as potassium chloride. The available commercial products form a variety of gels: gels that are clear or turbid, rigid or elastic, tough or tender, heat-stable or thermally reversible, and do or do not undergo syneresis. Carrageenan gels do not require refrigeration because they do not melt at room temperature. They are freeze–thaw stable.

A useful property of carrageenans is their reactivity with proteins, particularly those of milk. κ-Type carrageenans complex with κ-casein micelles of milk, forming a weak, thixotropic, pourable gel. The thickening effect of κ-carrageenans in milk is 5–10 times greater than it is in water. This property is used in the preparation of chocolate milk, in which the thixotropic gel structure prevents settling of cocoa particles. Such stabilization requires only about 0.025% gum. This property is also utilized in the preparation of ice cream, evaporated milk, infant formulas, freeze–thaw stable whipped cream, and emulsions in which milk fat is replaced with a vegetable oil.

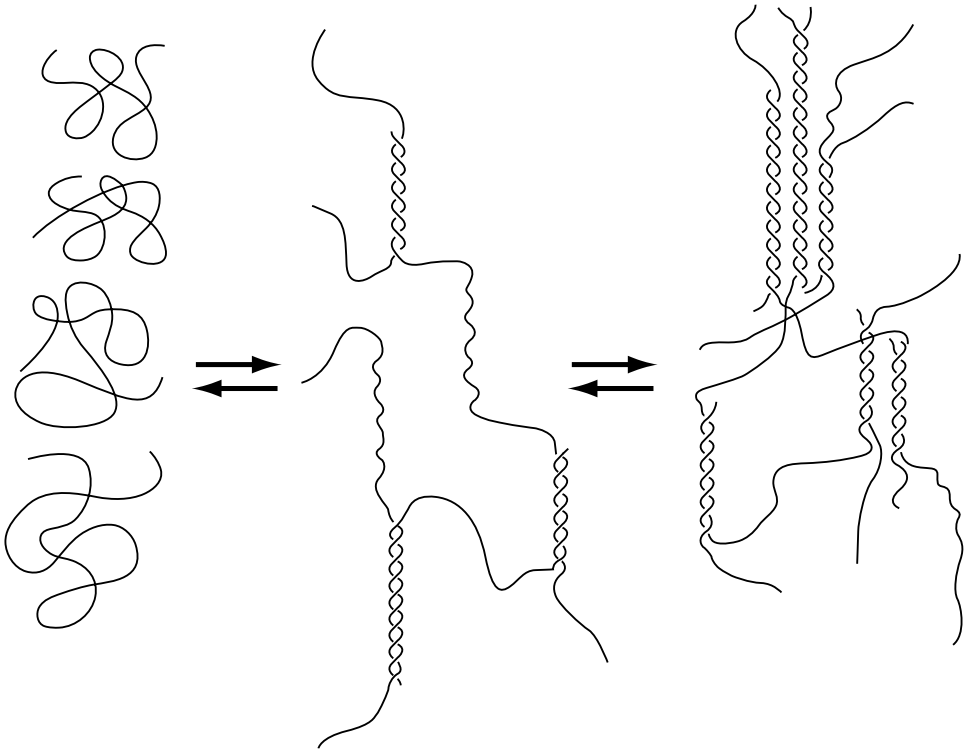

FIGURE 3.51 A representation of the hypothesized mechanism of gelation of κ- and ι-type carrageenans. In a hot solution, the polymer molecules are in a coiled state. As the solution is cooled, they intertwine in double-helical structures. As the solution is cooled further, the double helices are believed to nest together with the aid of potassium or calcium ions.

The synergistic effect between κ-carrageenan and LBG (Figure 3.49) produces gels with greater elasticity and gel strength, and with less syneresis than gels made with potassium κ-carrageenate alone. As compared to κ-type carrageenan alone, the κ-type carrageenan–LBG combination provides greater stabilization and air bubble retention (overrun) in ice cream, but also a little too much chewiness, so guar gum is added to soften the gel structure.

Cold hams and poultry rolls take up 20–80% more brine when they contain 1–2% of a κ-type carrageenan. Improved slicing also results. Carrageenan coatings on meats can serve as a mechanical protection and a carrier for seasonings and flavors. Carrageenan is sometimes added to meat analogs made from casein and vegetable proteins. Carrageenan is used to hold water and maintain water content, and therefore, to maintain softness of meat products, such as wieners and sausages, during the cooking operation. Addition of a κ- or ι-type carrageenan in the Na^+ form or processed Euchema seaweed/Philippine natural grade (PES/PNG) carrageenan (see next paragraph) to low-fat ground beef improves texture and hamburger quality. Normally, fat serves the purpose of maintaining softness, but because of the binding power of carrageenan for protein and its high affinity for water, carrageenans can be used to replace in part this function of natural animal fat in lean products.

Also prepared and used is an alkali-modified seaweed flour that was formerly called PES or PNG carrageenan, but is now often just called carrageenan. To prepare PES/PNG carrageenan, red seaweed is treated with a potassium hydroxide solution. Because the potassium salts of the types of carrageenans found in these seaweeds are insoluble, the carrageenan molecules are not solubilized and not extracted out. Primarily low-molecular-weight soluble components are removed

from the plants during this treatment. The remaining seaweed is dried and ground to a powder. PES/PNG carrageenan is, therefore, a composite material that contains not only the molecules of carrageenan that would be extracted with dilute sodium hydroxide, but also other cell wall materials.

Two other food gums, agar and furcellaran (also called Danish agar), also come from red seaweeds and have structures and properties that are closely related to those of the carrageenans. Like gellan (Section 3.3.13), the primary use of agar is in bakery mixes to which it is added to hold moisture in the final product without increasing the viscosity of the initial dough or batter (because it is not soluble in room-temperature water).

3.3.11 Algins [11,34]

Commercial algin is a salt, most often the sodium salt, of a linear poly(uronic acid), alginic acid, obtained from brown seaweeds (Table 3.5). Alginic acid is composed of two monomeric units, β-D-mannopyranosyluronic acid and α-L-gulopyranosyluronic acid units. These two monomers occur in homogeneous regions (composed exclusively of one unit or the other) and in regions of mixed units. Segments containing only D-mannuronopyranosyl units are referred to as M-blocks and those containing only L-guluronopyranosyl units as G-blocks. D-Mannuronopyranosyl units are in the $^4C_1$ conformation, while L-guluronopyranosyl units are in the $^1C_4$ conformation (see Section 3.1.2, Figure 3.52), which gives the different blocks quite different chain conformations. M-Block regions are flat and ribbon-like, similar to the conformation of cellulose (see Section 3.3.7) because of the equatorial → equatorial bonding. G-Block regions have a pleated (corrugated) conformation as a result of its axial → axial glycosidic bonds. Different percentages of the different block segments cause algins (alginates) from different seaweeds to have different properties. Algins with greater G-block contents produce gels of higher strength.

Solutions of sodium alginates are highly viscous. The calcium salt of alginates is insoluble. Insolubility results from interactions between calcium ions and the G-block regions of the chain. The holes formed between two G-block chains are cavities that bind calcium ions. The result is a junction zone that has been called an "egg box" arrangement with the calcium ions being likened to

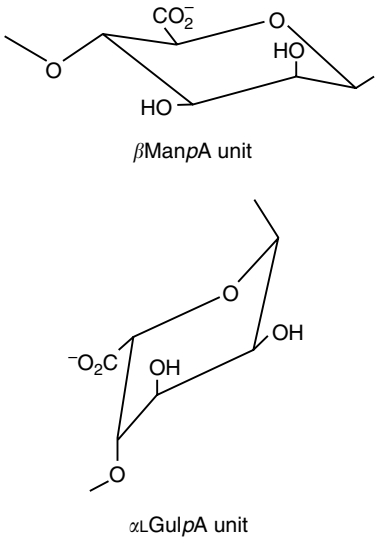

FIGURE 3.52 Units of β-D-mannopyranosyluronic acid (βMan*p*A) in the $^4C_1$ conformation and α-L-gulopyranosyluronic acid (αLGul*p*A) in the $^1C_4$ conformation.

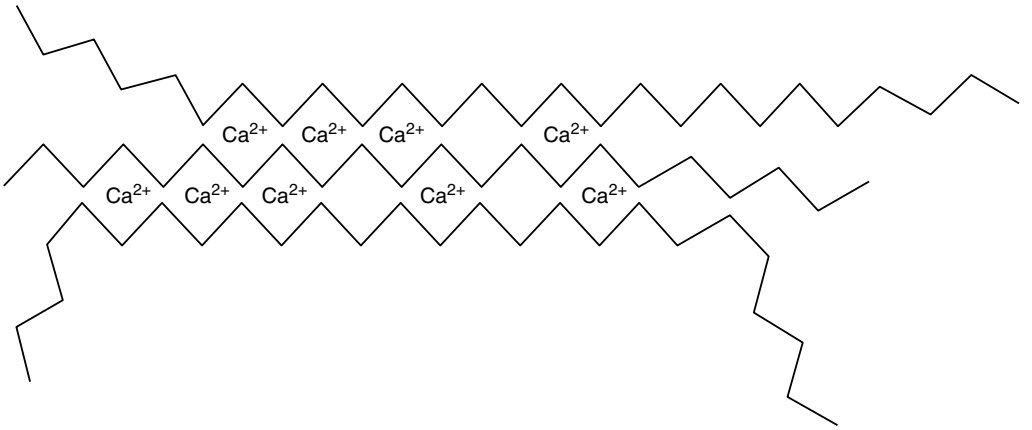

FIGURE 3.53 A representation of the proposed formation of a junction between G-block regions of three alginate molecules promoted by calcium ions.

eggs in the pockets of an egg carton (Figure 3.53). The strength of the gel depends on the content of G-blocks in the alginate used and the concentration of calcium ions.

Propylene glycol alginates are made by reacting moist alginic acid with propylene oxide to produce a partial ester with 50–85% of the carboxyl groups esterified. Solutions of PGAs are much less sensitive to low pH values and polyvalent cations, including calcium ions and proteins, than are solutions of nonesterified alginates, because esterified carboxyl groups cannot ionize. Also, the propylene glycol group introduces a "bump" on the chain that prevents close association of chains. Therefore, PGA solutions are stable. Because of its tolerance to calcium ions, PGAs can be used in dairy products. The hydrophobic propylene glycol groups also give the molecule mild interfacial activity, that is, foaming, emulsifying, and emulsion-stabilizing properties. PGA is used when stability to acid, nonreactivity with calcium ions (e.g., in milk products), or its surface active property is desired. Accordingly, it finds use as a thickener in salad dressings (Table 3.5). In low-calorie dressings, it is often used in conjunction with xanthan (Section 3.3.9).

Alginate salts are most often used as food ingredients because of their ability to form gels. However, they can be used to provide high viscosity at low concentrations and are particularly effective when a low concentration of calcium ions is present. If PGA is used, some calcium ion crosslinking of chains still occurs through the remaining carboxylate groups and results in thickening of solutions (rather than gelling).

Calcium alginate gels are obtained by diffusion setting, internal setting, and setting by cooling. Diffusion setting can be used to prepare structured foods. A good example is the structured pimento strip. In the production of pimento strips for stuffing green olives, pimento puree is first mixed with water containing a small amount of guar gum as an immediate thickener, and then with sodium alginate. The mixture is pumped onto a conveyor belt and gelled by addition of calcium ions. The set sheet is cut into thin strips and stuffed into olives. Internal setting for fruit mixes, purees, and fruit analogs involves a slow release of calcium ions within the mixture. The slow release is obtained by the combined action of a slightly soluble organic acid and a sequestrant on an insoluble calcium salt. Setting by cooling involves mixing the components required to form a gel at a temperature above the gel's melting temperature and allowing the mixture to set on cooling. Alginate gels are reasonably heat stable and show little or no syneresis. Unlike gelatin gels, alginate gels are not thermoreversible and, like carrageenan gels, do not require refrigeration and can be used as dessert gels that do not melt, even at high ambient temperatures; but as a result, they do not melt in the mouth like gelatin gels.

Alginic acid, that is, an alginate solution whose pH has been lowered, with and without addition of calcium ions, is employed in the preparation of soft, thixotropic, nonmelting gels (Table 3.5).

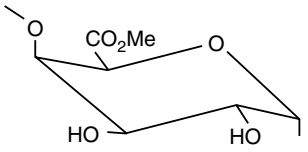

FIGURE 3.54 The most prevalent monomeric unit of an HM pectin.

3.3.12 Pectins [10,49]

Commercial pectins are galacturonoglycans (poly[α-D-galactopyranosyluronic acids]) with various contents of methyl ester groups (Table 3.5). The native molecules present in the cell walls and intercellular layers of all land plants, from which commercial pectins are obtained, are more complex molecules that are converted into methyl esterified galacturonoglycans during extraction with acid. Commercial pectin is obtained from citrus peel and apple pomace. Pectin from lemon and lime peel generally is the easiest to isolate and is of the highest quality. Pectins have a unique ability to form spreadable gels in the presence of sugar and acid or in the presence of calcium ions and are used primarily in these types of applications.

The compositions and properties of pectins vary with source, the processes used during preparation, and subsequent treatments. During extraction with mild acid, some hydrolytic depolymerization and hydrolysis of methyl ester groups occurs. Therefore, the term pectin denotes a family of compounds. The term pectin is usually used in a generic sense to designate those water-soluble poly(galacturonic acid) (galacturonoglycan) preparations of varying methyl ester contents and degrees of neutralization that are capable of forming gels. In all natural pectins, some of the carboxyl groups are in the methyl ester form. Depending on the manufacturing conditions, the remaining free carboxylic acid groups may be partly or fully neutralized, that is, partly or fully present as sodium, potassium, or ammonium carboxylate groups. Typically, they are present in the sodium salt form.

By definition, preparations in which more than half of the carboxyl groups are in the methyl ester form ($-COOCH_3$) are classified as high-methoxyl (HM) pectins (Figure 3.54); the remainder of the carboxyl groups will be present as a mixture of free acid ($-COOH$) and salt (e.g., $-COO^-Na^+$) forms. Preparations in which less than half of the carboxyl groups are in the methyl ester form are called low-methoxyl (LM) pectins. The percentage of carboxyl groups esterified with methanol is the degree of esterification (DE) or degree of methylation (DM). Treatment of a pectin preparation with ammonia (often dissolved in methanol) converts some of the methyl ester groups into carboxamide groups (15–25%). In the process, a LM pectin (by definition) is formed. These products are known as amidated LM pectins.

The principal and key feature of all pectin molecules is a linear chain of (1→4)-linked α-D-galactopyranosyluronic acid units. Neutral sugars, primarily L-rhamnose, are also present. In citrus and apple pectins, the α-L-rhamnopyranosyl units are inserted into the polysaccharide chain at rather regular intervals. The inserted L-rhamnopyranosyl units may provide the necessary irregularities in the structure required to limit the size of the junction zones and effect gelation (as opposed to precipitation/complete insolubility). At least some pectins contain covalently attached, highly branched arabinogalactan chains and/or short side chains composed of D-xylosyl units. The presence of side chains may also be a factor that limits the extent of chain association. Junction zones are formed between regular, unbranched pectin chains when the negative charges on the carboxylate groups are removed (addition of acid), hydration of the molecules is reduced (by addition of a cosolute, almost always sugar, to a solution of HM pectin), and/or when polymer chains are bridged by calcium cations.

High-methoxyl pectin solutions gel when sufficient acid and sugar is present. As the pH of a pectin solution is lowered, the highly hydrated and charged carboxylate groups are converted into uncharged, only slightly hydrated carboxylic acid groups. As a result of losing some of their charge

and hydration, the polymer molecules can now associate over a portion of their length, forming junctions and a network of polymer chains that entraps the aqueous solution of solute molecules. Junction zone formation is assisted by the presence of a high concentration (~65%, at least 55%) of sugar, which competes with the pectin molecules for the water molecules and reduces hydration of the chains, allowing them to interact with one another.

Low-methoxyl pectin solutions gel only in the presence of divalent cations that provide crossbridges. Increasing the concentration of divalent cations (only calcium ion is used in food applications) increases the gelling temperature and gel strength. The same general egg box model used to describe the formation of calcium alginate gels (Section 3.3.11) is used to explain gelation of solutions of LM (both standard and amidated) pectins upon addition of calcium ions. Since it does not require sugar for gelation, LM pectin is used to make low-sugar jams, jellies, and marmalades.

3.3.13 Gellan [44]

Gellan, known commercially as gellan gum (Table 3.5), is an extracellular, anionic polysaccharide produced by the bacterium *Sphingomonas elodea.* The gellan molecule is linear and is composed of β-D-glucopyranosyl, β-D-glucuronopyranosyl, and α-L-rhamnopyranosyl units in a molar ratio of 2:1:1. Native gellan (also called high-acyl gellan) contains two ester groups, an acetyl group and a glyceryl group, both on the same glucosyl unit. On average, there is one glycerate ester group per tetrasaccharide repeat unit and one acetate ester group for every two repeat units.

Some gellan is de-esterified by treatment with alkali. Removal of the acyl groups has a dramatic effect on the gel properties of gellan. The de-esterified form is known as low-acyl gellan. Its tetrasaccharide repeat unit structure is $\rightarrow$4)-αLRha*p*-(1$\rightarrow$3)-βGlc*p*-(1$\rightarrow$4)-βGlc*p*A-(1$\rightarrow$4)-βGlc*p*-(1$\rightarrow$. Three basic forms of the gum are available: high-acyl (native), low-acyl clarified, and low-acyl unclarified. The majority of gellan used in food products is the low-acyl, clarified type. Blending of high- and low-acyl types results in products with intermediate properties.

Gellan can form gels with both monovalent and divalent cations, divalent cations (Ca^{2+}) being about ten times more effective. Gels can be formed with as little as 0.05% gum (99.95% water). Gelation is often affected by cooling a hot solution containing the required cation. Shearing during cooling of a hot gellan solution prevents the normal gelation mechanism from occurring and produces a smooth, homogeneous, thixotropic fluid (a pourable gel) that stabilizes emulsions and suspensions very effectively. Gentle agitation of a weak gellan gel will also disrupt the gel structure and turn the gel into a smooth, pourable, thixotropic fluid with excellent emulsion and suspension stabilizing properties.

The low-acyl types of gellan form firm, brittle, nonelastic gels (with textures similar to those of gels made with agar and κ-carrageenan). The high-acyl (native) type forms soft, elastic, non-brittle gels (with textures similar to those made with mixtures of xanthan and LBG). A range of intermediate gel textures can be achieved by mixing the two basic types of gellan.

When gellan is used as an ingredient in bakery mixes, it does not hydrate appreciably at room temperature, nor increase the viscosity of the batter. It does, however, hydrate upon heating and holds moisture in the baked product. Gellan is used in formulating nutrition bars because of its moisture retaining ability. The ability of its solutions to suspend at low concentration (without producing high viscosity) makes it useful in nutritional and diet beverages.

3.3.14 Curdlan [37]

Curdlan is a bacterial polysaccharide produced by *Agrobacterium biovar* (Table 3.5). It is a 1,3-linked β-glucan that has the unique property of forming gels when solutions of it are heated. Curdlan forms two types of gels that differ in thermoreversibility. A thermally reversible gel is formed when solutions of curdlan are heated to about 65°C, then cooled to about 60°C. However, when curdlan solutions are heated to about 80°C, a strong, thermally irreversible gel forms, that is, a solution is

not reformed upon cooling. Gel strength continues to increase with increasing temperature up to about 130°C.

3.3.15 Gum Arabic [21,63]

When the bark of some trees and shrubs is injured, the plants exude a sticky material that hardens to seal the wound and give protection from infection and desiccation. Such exudates are commonly found on plants that grow in semiarid conditions. Since they are sticky when freshly exuded, dust, insects, bacteria, and/or pieces of bark adhere to the exudate tears (as they are called). Gum arabic (gum acacia), gum karaya, and gum ghatti are exudates of trees; gum tragacanth is the exudate of a shrub. Of the exudate gums, only gum arabic is a major food gum today.

Gum arabic (gum acacia) is an exudate of acacia trees, of which there are many species distributed over tropical and subtropical regions (Table 3.5). The most important growing areas for the species that give the best gum are Sudan and Nigeria. Purified, spray-dried forms of gum arabic are commonly used.

Gum arabic is a heterogeneous material, but generally consists of two primary fractions. One, which accounts for about 70% of the gum, is composed of polysaccharide chains with little or no protein. The other fraction contains molecules of higher molecular weight that have protein as an integral part of their structures. The protein–polysaccharide fraction is itself heterogeneous with respect to protein content. The polysaccharide structures are covalently attached to the protein component by linkage to hydroxyproline and, perhaps, serine units, the two predominant amino acids in the polypeptide. The overall protein content is about 2 wt%, but fractions may contain as much as 25 wt% protein.

The polysaccharide structures, both those attached to protein and those that are not, are highly branched, acidic arabinogalactans with the following approximate composition: D-galactose, 44%; L-arabinose, 24%; D-glucuronic acid, 14.5%; L-rhamnose, 13%; 4-*O*-methyl-D-glucuronic acid, 1.5%. They contain main chains of (1→3)-linked β-D-galactopyranosyl units having two- to four-unit side chains consisting of (1→3)-linked β-D-galactopyranosyl units joined to it by (1→6) linkages. Both the main chain and the numerous side chains have attached α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-glucuronopyranosyl, and 4-*O*-methyl-β-D-glucuronopyranosyl units. The uronic acid units occur most often as nonreducing end units.

Gum arabic dissolves easily when stirred in water. It is unique among the food gums, except for gums that have been depolymerized to produce low-viscosity types, because of its high solubility and the low viscosity of its solutions. Solutions of 50% concentration can be made. Above this concentration, dispersions are somewhat gel-like.

Gum arabic is both a fair emulsifying agent and a very good emulsion stabilizer for flavor oil-in-water emulsions. It is the gum of choice for emulsification of citrus oils, other essential oils, and imitation flavors used as concentrates for soft drinks and baker's emulsions. In the United States, the soft drink industry consumes about 30% of the gum supply as an emulsifier and stabilizer. For a gum to have an emulsion stabilizing effect, it must have anchoring groups with a strong affinity for the surface of the oil and a molecular size large enough to cover the surfaces of dispersed droplets. Gum arabic has surface activity and forms a thick, sterically stabilizing, macromolecular layer around oil droplets. Emulsions made with flavor oils and gum arabic can be spray dried to produce dry flavor powders that are nonhygroscopic and in which the flavor oil is protected from oxidation and volatalization. Rapid dispersion and release of flavor without affecting product viscosity are other attributes. These stable flavor powders are used in dry package products such as beverage, cake, dessert, pudding, and soup mixes.

Another important characteristic of gum arabic is its compatibility with high concentrations of sugar. Therefore, it finds widespread use in confections with a high sugar content and a low water content. More than half the world's supply of gum arabic is used in confections such as caramels, toffees, jujubes, and pastilles. In confections it prevents sucrose crystallization, emulsifies and

distributes fatty components, and helps prevent bloom (the surface whitening caused by polymorphic transitions of lipids). Another use is as a component of the glaze or coating of pan-coated candies.

3.3.16 Inulin and Fructooligosaccharides [15–17,19,55]

Inulin (Table 3.5) occurs naturally as a storage carbohydrate in thousands of plant species, including onion, garlic, asparagus, and banana. The primary commercial source is chicory (*Chicorium intybus*) root. Some is also obtained from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers.

Inulin is composed of β-D-fructofuranosyl units linked $2\rightarrow1$. The polymer chains are often, but not always (because of degradation, either natural or during isolation), terminated at the reducing end with a sucrose unit. Inulin's DP rarely, if ever, exceeds 60. It occurs in plants together with fructooligosaccharides, giving an overall DP in the range of 2–60.

Molecules containing furanosyl units, such as molecules of inulin and sucrose, undergo acid-catalyzed hydrolysis much more easily than do those containing pyranosyl units. Inulin is a storage, that is, a reserve food, polysaccharide, so it is seemingly apparent that, at any time, molecules in various stages of synthesis and, perhaps, breakdown are present. As a result, inulin preparations are mixtures of fructooligosaccharides and small polysaccharides molecules.

Inulin is often deliberately depolymerized into fructooligosaccharides. Both inulin and the fructooligosaccharide products produced from it are prebiotics. (Prebiotics are nondigestible food ingredients that have a beneficial effect on the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already present in the colon. Prebiotics are most often used for the nutritional/health benefits they impart.)

Aqueous solutions of inulin can be made at concentrations as high as 50%. When hot solutions of inulin of concentrations greater than 25% are cooled, thermoreversible gels are formed. Inulin gels are described as particle gels (especially after shearing) with a creamy, fat-like texture. Hence, inulin can be used as a fat mimetic in reduced-fat products. It improves the texture and mouthfeel of low-fat ice creams and sauces. Inulin is an ingredient in nutrition, breakfast, meal replacement bars, sports/energy bars, soy beverages, and vegetable patties.

Neither inulin nor fructooligosaccharides are digested by enzymes in either the stomach or small intestine. Therefore, they are components of dietary fiber (Section 3.4). They have a glycemic index of zero, that is, they raise neither the glucose nor the insulin levels in the blood.

3.4 DIETARY FIBER AND CARBOHYDRATE DIGESTIBILITY [2,8,9,15,16,31,35,41,53,60,62,64,67]

Carbohydrates have always been the principal source of metabolic energy for humans and the means for maintaining health of the human gastrointestinal tract. Carbohydrates are also the principal providers of the bulk and body of food products.

Plant cell wall materials, primarily cellulose, other nonstarch polysaccharides and lignin, are components of dietary fiber. The only common feature of these polymers is that they are nondigestible, which is the principal criterion for being classified as a component of dietary fiber. Therefore, not only do natural components of foods contribute dietary fiber, so also do gums that are added to provide the functionalities described in Sections 3.3.7–3.3.16. The definition of dietary fiber also includes substances other than polymers. The key characteristic is that the substance not be digested in the human small intestine, so nondigestible oligosaccharides, for example, raffinose and stachyose (Section 3.2.3), are included as dietary fiber substances.

Oligo- and polysaccharides may be digestible (most starch-based products), partially digestible (retrograded amylose, so-called resistant starch), or nondigestible (essentially all other polysaccharides). When digestive hydrolysis to monosaccharides occurs, the products of digestion are absorbed

and catabolized. (Only monosaccharides can be absorbed through the wall of the small intestine, and only D-glucose is produced by digestion of polysaccharides in humans because only starches can be digested.) Those carbohydrates not digested to monosaccharides by human enzymes in the small intestine (all others except sucrose, lactose, and products, such as maltodextrins, made from starch) may be metabolized by microorganisms in the large intestine, producing low-molecular-weight acids that are partially absorbed and catabolized for energy. Therefore, carbohydrates of all molecular sizes may be caloric, partially caloric, or essentially noncaloric.

The most common bulking agents in natural food are remnants of plant cells that are resistant to hydrolysis by enzymes in the digestive tract. This material includes cellulose, hemicelluloses, pectin, and lignin. Dietary fiber is important in nutrition because it maintains the normal functioning of the gastrointestinal tract. Dietary fiber increases intestinal and fecal bulk, which lowers intestinal transit time and helps prevent constipation. Its presence in foods induces satiety at meal time. Nutritionists set requirements of dietary fiber at 25–50 g per day. Insoluble fiber is claimed to decrease blood cholesterol levels, lessening the chance of heart disease. It also reduces the chances of colonic cancer, probably due to its sweeping action.

Soluble gums have similar effects in the gastrointestinal tract and on the level of cholesterol in blood, but to different extents. Some gums that have been specifically examined in this regard are pectin, guar gum, xanthan, and hemicelluloses (e.g., guar gum ingested at a rate of 5 g/day results in a lowering of the hyperglycemic spike, a 13% lowering of serum cholesterol, and no decrease in the high-density lipoprotein [HDL] fraction, the beneficial cholesterol carrier.) In addition to cereal brans, kidney and navy beans are especially good sources of dietary fiber. A product based on psyllium seed hulls has high water-binding properties, leading to rapid transit time in the gastrointestinal tract, and is used to prevent constipation. A product with a MC base is sold for the same purpose.

The starch polysaccharides are the only polysaccharides that can be hydrolyzed by human enzymes. They, of course, provide D-glucose that is absorbed by microvilli of the small intestine to supply the principal metabolic energy of humans. Other polysaccharides consumed normally as natural components of edible vegetables, fruits, and other plant materials and those food gums added to prepared food products are not digested in the stomach or small intestine of humans, but they pass into the large intestine (colon) with little or no change. (The acidity of the stomach is neither strong enough, nor is the residence time of polysaccharides in the stomach sufficiently long to cause significant chemical cleavage.) When the undigested polysaccharides reach the large intestine, they come into contact with normal intestinal microorganisms, some of which produce enzymes that catalyze hydrolysis of certain polysaccharides or certain parts of polysaccharide molecules. The consequence of this is that polysaccharides not cleaved in the upper intestinal tract may be broken down and utilized by the bacteria within the large intestine.

Sugars that are split from the polysaccharide chain are used by the microorganisms of the large intestine as energy sources in anaerobic fermentation pathways that produce lactic, propionic, butyric, and valeric acids. These short-chain acids can be absorbed through the intestinal wall and metabolized, primarily in the liver. In addition, a small, though significant in some cases, fraction of the released sugars can be taken up by the intestinal wall and transported to the portal blood stream where they are conveyed to the liver and metabolized. It is calculated that, on average, ~7% of human energy is derived from sugars split from polysaccharides by microorganisms in the large intestine or from the short-chain acids produced from them via anaerobic fermentation pathways. The extent of polysaccharide cleavage depends on the abundance of the particular organism(s) producing the specific enzymes required. Thus, when changes occur in the type of polysaccharide consumed, utilization of the polysaccharide by colonic microorganisms may be temporarily reduced until organisms capable of splitting the new polysaccharide proliferate.

Some polysaccharides survive almost intact during their transit through the entire gastrointestinal tract. These, plus larger segments of other polysaccharides, give bulk to the intestinal contents and lower the transit time. They can be a positive factor in health through a lowering of blood cholesterol concentration, perhaps by sweeping out bile salts and reducing their chances for reabsorption from

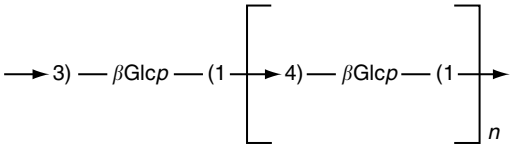

FIGURE 3.55 Representative structure (shorthand notation) of a segment of oat and barley β-glucans where n usually is 1 or 2, but occasionally may be larger.

the intestine. In addition, the presence of large amounts of hydrophilic molecules maintain sufficient water content in the intestinal contents that results in stool softness and consequent easier passage through the large intestine.

One natural component of dietary fiber is a water-soluble polysaccharide, β-glucan, that is present in oat and barley brans. Oat β-glucan has become a commercial food ingredient because it has been shown to be effective in reducing the level of serum cholesterol. The oat β-glucan molecule is a linear chain of β-D-glucopyranosyl units. About 70% are linked as (1→4) and about 30% as (1→3). The (1→3) linkages occur singly and are separated by sequences of two or three (1→4) linkages. Thus, the molecule is composed of (1→3)-linked β-cellotriosyl [→3)-βGlc*p*-(1→4)-βGlc*p*-(1→4)-βGlc*p*-(1→] and β-cellotetraosyl units (Figure 3.55). Such (1→4,1→3)-β-glucans are often called mixed linkage β-glucans.

When taken orally in foods, β-glucans reduce postprandial serum glucose levels and the insulin response, that is, they moderate the glycemic response, in both normal and diabetic human subjects. This effect seems to be correlated with viscosity. They also reduce serum cholesterol concentrations in rats, chickens, and humans. These physiological effects are typical of those of soluble dietary fiber. Other soluble polysaccharides have similar effects but to differing degrees.

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4 Lipids

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CONTENTS

4.1 INTRODUCTION

Lipids are a broad group of chemically diverse compounds that are soluble in organic solvents. Food lipids are generally referred to as fats (solid) or oils (liquid) indicating their physical state at ambient temperatures. Food lipids are also classified as nonpolar (e.g., triacylglycerol and cholesterol) and polar lipids (e.g., phospholipids) to indicate differences in their solubility and functional properties. Polar lipids often contain a hydrophilic "head" group that has a high affinity for water attached to a lipophilic "tail" group that has a high affinity for oil [1]. These surface-active lipids may alter the properties of foods through a variety of physicochemical mechanisms, including adsorbing to interfaces, stabilizing particles, interacting with biopolymers, and modifying crystal nucleation, growth, and structure [1–5] (see Chapter 13 for more details).

The total lipid content and the lipid composition of foods can vary tremendously. Since food lipids play an important role in food quality by contributing to attributes such as texture, flavor, nutrition, and caloric density, the manipulation of these important food components has been a major emphasis in food product development research over the past few decades. This research has focused

on the alteration of lipid composition to change texture, alter fatty acid and cholesterol composition, decrease total fat, alter bioavailability, and make lipids more oxidatively stable. In addition, the physical stability of lipids is important in food quality since many lipids exist as dispersions/emulsions that are thermodynamically unstable. In order to make changes in lipid composition while ensuring the production of high quality foods, a fundamental understanding of the chemical and physical properties of lipids is critical. This chapter will focus on the chemical composition of lipids, their physical properties and crystallization behavior, methods to modify the fatty acid and triacylglycerol composition and thus the physicochemical properties of lipids, propensity to undergo oxidative deterioration, and the role of lipids in health and disease. Information on analytical methods for food lipids is provided elsewhere [6,7].

4.2 MAJOR LIPID COMPONENTS

The following section is a brief description of the nomenclature of the major classes of food lipids. For more information of lipid nomenclature see O'Keefe [8] or the web page of the International Union of Pure and Applied Chemists (IUPAC), http://www.chem.qmul.ac.uk/iupac/lipid.

4.2.1 Fatty Acids

The major components of lipids are fatty acids, compounds that contain an aliphatic chain with a carboxylic acid group. Most natural fatty acids have an even number of carbons in a straight chain because of the biological process of fatty acid elongation where two carbons are added at a time. Exceptions of fatty acids with odd carbon numbers and branched chains can be found in sources such as microorganisms and dairy fats. The majority of fatty acids in nature range from 14 to 24 carbons. While some fats contain fatty acids with <14 carbons, significant levels of short-chain fatty acids are mainly found in tropical oils and dairy fats. Fatty acids are generally classified as either saturated or unsaturated, with unsaturated fatty acids containing double bonds. Fatty acids can be described by systematic, common, and abbreviated names.

4.2.1.1 Nomenclature of Saturated Fatty Acids

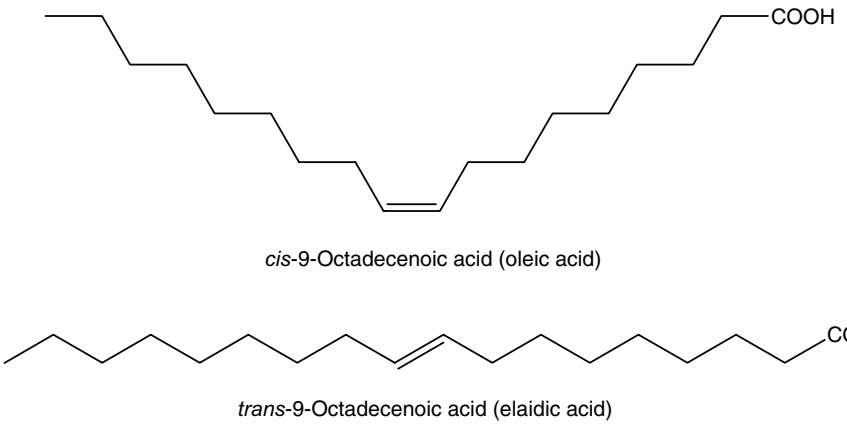
The IUPAC have standardized systematic descriptions of fatty acids. The IUPAC system names the parent hydrocarbon of the fatty acid on the basis of the number of carbons (e.g., ten carbons would be decane). Since fatty acids contain a carboxylic acid group, the terminal *e* in the hydrocarbon's name is replaced by *oic* (e.g., decanoic; Table 4.1). Common names exist for most of the even number and many of the odd number fatty acids (Table 4.1). Many of the common names originate from the source that the fatty acid was commonly or traditionally isolated (e.g., palmitic acids and palm oil). A numerical system can be used for abbreviated names. The first number in this system designates the number of carbons in the fatty acids while the second number designates the number of double bonds (e.g., hexadecanoic = palmitic = 16:0). Obviously, this second number will always be zero for the saturated fatty acids.

4.2.1.2 Nomenclature of Unsaturated Fatty Acids

Fatty acids that contain double bonds in their aliphatic chain are referred to as unsaturated fatty acids. In the IUPAC system, the *anoic* designation is changed to *enoic* to designate the presence of a double bond (Table 4.1). On the basis of the number of double bonds, the terms *di-*, *tri-*, *tetra-*, and so on are added. Common names also exist for the unsaturated fatty acids (with the exception of some of the long-chain polyunsaturated fatty acids) and the numerical abbreviation system is similar to the saturated fatty acids with the second number indicating the number of double bonds (e.g., octadecadienoic = 18:2). The positions of the double bonds in the IUPAC system are numbered by the delta (Δ) system that indicates the position of the double bonds from the carboxylic acid end of the fatty acid. For example, oleic acid, which has 18 carbons and one double bond, would

TABLE 4.1
Systematic, Common, and Numerical Names for Fatty Acids Found in Foods

Systematic Name	Common Name	Numerical Abbreviation
	Saturated fatty acids	
Hexanoic	Caproic	6:0
Octanoic	Caprylic	8:0
Decanoic	Capric	10:0
Dodecanoic	Lauric	12:0
Tetradecanoic	Myristic	14:0
Hexadecanoic	Palmitic	16:0
Octadecanoic	Stearic	18:0
	Unsaturated fatty acids	
cis-9-Octadecenoic	Oleic	18:1 Δ9
cis-9, *cis*-12-Octadecadienoic	Linoleic	18:2 Δ9
cis-9, *cis*-12, *cis*-15-Octadecatrienoic	Linolenic	18:3 Δ9
cis-5, *cis*-8, *cis*-11, *cis*-14-Eicosatetraenoic	Arachidonic	20:4 Δ5
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17-Eicosapentaenoic	EPA	20:5 Δ5
cis-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19-Docosahexaenoic	DHA	22:6 Δ4



cis-9-Octadecenoic acid (oleic acid)

trans-9-Octadecenoic acid (elaidic acid)

FIGURE 4.1 Differences between *cis* and *trans* double bonds in unsaturated fatty acids.

be 9-octadecenoic acid and linoleic acid, which has 18 carbons and two double bonds, would be 9, 12-octadecadienoic acid. An alternative numbering system that indicates the position of the double bonds from the methyl end of the fatty acids is known as the omega (ω) system (sometimes given a shorthand notation of "*n*"). The ω system is sometimes useful because it can group fatty acids on the basis of their biological activity and biosynthetic origin since many enzymes recognize fatty acids from the free methyl end of the molecule when it is esterified to glycerol. For instance, the ω-3 fatty acids often have similar bioactivity in their ability to decrease blood triacylglycerol levels [9].

The natural configuration of double bonds in unsaturated fatty acids is the *cis* configuration. In the *cis* configuration, the carbons of the aliphatic chain are on the same side of the double bond while *trans* double bonds would have the carbons on opposite sides (Figure 4.1). Double bonds in polyunsaturated fatty acids (greater than two double bonds) are most commonly in a methylene-interrupted configuration often termed the pentadiene system. In a pentadiene system, the two double bonds would be at carbons 1 and 4. In other words, the double bonds are not conjugated but instead

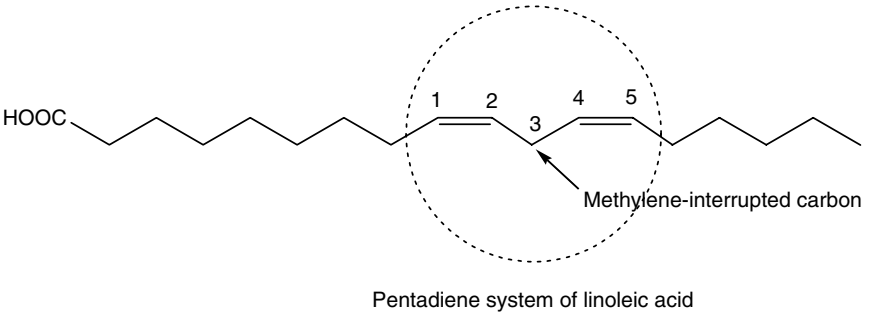


FIGURE 4.2 The pentadiene systems of the polyunsaturated fatty acid, linoleic acid.

are separated by a methylene-interrupted carbon (Figure 4.2). This means that the double bonds of most unsaturated fatty acids are three carbons apart (e.g., 9, 12, 15 octadecatrienoic). It is therefore possible to predict the position of all the double bonds in most natural unsaturated fatty acids if the location of the first double bond is known. This is why the numerical abbreviation system will sometimes only give the number of double bonds and the position of the first double bond (e.g., 9, 12, 15 octadecatrienoic = 18:3, $\Delta 9 = 18{:}3$, $\omega 3$).

The presence of double bonds influences the melting point of the fatty acids. Double bonds in the *cis* configuration will cause the fatty acid to arrange in a bent configuration. Thus, unsaturated fatty acids are not linear making it difficult for them to orient themselves into tight packing configurations. Because of stearic hindrance to packing, van der Waals interactions between unsaturated fatty acids are relatively weak; therefore, they exist mainly in the liquid state at room temperature; that is, their melting point/solidification temperature is relatively low. As more double bonds are added, the molecule becomes more bent, the van der Waals interactions decrease further, and the melting point decreases. Fatty acids with double bonds in the *trans* configuration are more linear than unsaturated fatty acids in the *cis* configuration. This results in tighter packing of the molecules and higher melting points. For example, the approximate melting point of stearic acid (octadecanoic) is 70°C, oleic acid (*cis*-9-octadecenoic) is 5°C, and elaidic acid (*trans*-9-octadecenoic) is 44°C [10].

4.2.2 Acylglycerols

Over 99% of the fatty acids found in plants and animals are esterified to glycerol. Free fatty acids are not common in living tissues because they are cytotoxic owing to their ability to disrupt cell membrane organization. Once fatty acids are esterified onto glycerol, their surface activity decreases, as does their cytotoxicity.

Acylglycerols can exist as mono-, di-, and triesters known as monoacylglycerols, diacylglycerols, and triacylglycerols, respectively. Triacylglycerols are the most common of the three in foods, although the mono- and diesters are sometimes used as food additives (e.g., emulsifiers). The central carbon of a triacylglycerol exhibits chirality if different fatty acids are present at the terminal carbons of the glycerol. Because of this, the three carbons on the glycerol portion of the triacylglycerol can be differentiated with stereospecific numbering (*sn*). If the triacylglycerol is shown in a planar Fischer projection, the carbons are numbered 1–3 from top to bottom.

Triacylglycerols can be named by several different systems. Triacylglycerols are often named using the common names of the fatty acids. If the triacylglycerol contains only one fatty acid (e.g., stearic acid abbreviated as St), it could be named tristearin, tristearate, glycerol tristearate, tristearoyl glycerol, StStSt, or 18:0–18:0–18:0. Triacylglycerols that contain different fatty acids are named differently depending on whether the stereospecific location of each fatty acid is known. The nomenclature for these heterogeneous triacylglycerols replaces the *-ic* at the end of the fatty acid name with *-oyl*. If the stereospecific location is not known, a triacylglycerol-containing

palmitic acid, oleic acid, and stearic acid would be named palmitoyl-oleoyl-stearoyl-glycerol. Alternatively, this triacylglycerol could be named palmito-oleo-stearin or glycerol-palmito-oleo-stearate. If the stereospecific location of the fatty acids is known, *sn-* is added to the name such as in 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol, *sn*-1-palmito-2-oleo-3-stearin, or *sn*-glycerol-1-palmito-2-oleo-3-stearate. If two of the fatty acids are identical, the naming can be shortened as 1,2-dipalmitoyl-3-stearoyl-*sn*-glycerol, *sn*-1,2-dipalmito-3-stearin, or *sn*-glycerol-1,2-dipalmito-3-stearate. Heterogeneous triacylglycerol can also be named using fatty acid abbreviations such as in PStO or 16:0–18:0–18:1 (stereospecific location unknown) or *sn*-PStO or *sn*-16:0–18:0–18:1 (stereospecific location known) for 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol.

4.2.2.1 Composition of Fats

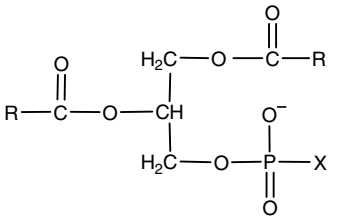
Food lipids contain a wide variety of fatty acid compositions as shown in Table 4.2. Several general trends can be seen among lipids. Most vegetable oils, especially those from oilseeds, are highly unsaturated and contain primarily fatty acids in the 18 carbon series. Oils high in oleic acid include olive and canola, oils high in linoleic include soybean and corn, and oils high in linolenic include linseed. Triacylglycerols from plant sources that contain high amounts of saturated fatty acids include cocoa butter and the tropical oils (e.g., coconut). Coconut and palm kernel oils are also unique in that they contain high amounts of the medium-chain fatty acids 8:0 to 14:0 with 12:0 predominating. The level of saturated fatty acids in fats and oils from animals is generally in the order of milk fat > sheep > beef > pig > chicken > turkey > marine fish with palmitic and stearic being the major saturated fatty acids. The fatty acid composition of animal fats are dependent on the digestive system of the animal with fat from nonruminants (e.g., poultry, pigs, and fish) being partially dependent on the fatty acid compositions of their diets. An example of this is pigs, such as Iberian hams, where dietary regimes are manipulated to produce lard with a high oleic acid composition. Among the nonruminants, triacylglycerols from marine animals are unique because they contain high amounts of the ω-3 fatty acids, eicosapentaenoic and docosahexaenoic. In sheep and cows, dietary fatty acids are subject to biohydrogenation by microbial enzymes in the rumen. This results in the conversion of unsaturated fatty acids into saturated fatty acids and can also produce fatty acids with conjugated double bonds such as conjugated linoleic acid (CLA). Since ruminants consume primarily lipids of plant origin where the fatty acids are primarily in the 18 carbon series, the end product of this biohydrogenation pathway is stearic acid. Thus, butter, beef fat, and sheep fat contain higher amounts of stearic acid than fats from nonruminants. Ruminal bacteria are also unique in that they can ferment carbohydrates to acetate and β-hydroxybutyrate. In the mammary gland, these substrates are converted to fatty acids to give butter fat a high concentration of saturated, short-chain (4:0 and 6:0) fatty acids that are not found in other food triacylglycerols. Ruminal bacteria also promote the formation of keto-, hydroxyl, and branched fatty acids. Because of the impact of ruminal bacteria on fatty acids, butter fat contains hundreds of different fatty acids.

The stereospecific location of fatty acids can also vary in food triacylglycerols. Triacylglycerols in some fats such as tallow (beef fat), olive oil, and peanut oil have most of their fatty acids evenly distributed among all three positions of glycerol. However, some fats can have very specific trends for the stereospecific location of fatty acids. Many triacylglycerols from plants sources have the (poly)unsaturated fatty acids concentrated at the *sn*-2 position. The best example of this is cocoa butter where over 85% of its oleic acid is *sn*-2, with palmitic and stearic acids being evenly distributed at *sn*-1 and *sn*-3. Triacylglycerols from some animal fats tend to have saturated fatty acids concentrated at *sn*-2. For instance, palmitic acid is primarily at the *sn*-2 position in milk fat and lard (pork fat). The stereospecific location of a fatty acid can be an important determinant on their impact in nutrition. When triacylglycerols are digested in the intestine, fatty acids from *sn*-1 to *sn*-3 are released by pancreatic lipase resulting in two free fatty acids and a *sn*-2 monoacylglycerol. If long-chain saturated fatty acids are at *sn*-1 and *sn*-3, their bioavailability is lower because the free fatty acids can form insoluble calcium salts on hydrolysis by pancreatic lipase. Thus, placement of long-chain saturated

TABLE 4.2
Fatty Acid Composition (Weight% of Total Fatty Acids) of Common Foods (Only the Major Fatty Acids in These Products Are Listed)

Food Lipid	4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1Δ9	18:0	18:1Δ9	18:2Δ9	18:3Δ9	20:5Δ5	22:6Δ4	Total Saturated	Crystal Habit
Olive							13.7	1.2	2.5	71.1	10.0	0.6			16.2	β
Canola							3.9	0.2	1.9	64.1	18.7	9.2			5.5	β
Corn							12.2	0.1	2.2	27.5	57.0	0.9			14.4	β
Soybean						0.1	11.0	0.1	4.0	23.4	53.2	7.8			15.0	β
Linseed							4.8		4.7	19.9	15.9	52.7			9.5	
Coconut		0.5	8.0	6.4	48.5	17.6	8.4		2.5	6.5	1.5				91.9	β'
Cocoa						0.1	25.8	0.3	34.5	35.3	2.9				60.4	β
Butterfat	3.8	2.3	1.1	2.0	3.1	11.7	26.2	1.9	12.5	28.2	2.9	0.5			62.7	β'
Beef fat				0.1	0.1	3.3	25.5	3.4	21.6	38.7	2.2	0.6			50.6	β'
Pork fat				0.1	0.1	1.5	24.8	3.1	12.3	45.1	9.9	0.1			38.8	β
Chicken					0.2	1.3	23.2	6.5	6.4	41.6	18.9	1.3			31.1	
Atlantic Salmon						5.0	15.9	6.3	2.5	21.4	1.1	0.6	1.9	11.9	23.4	β'
Chicken Eggs						0.3	22.1	3.3	7.7	36.6	11.1	0.3			30.1	

Source: All fatty acid compositions are adapted from White, P.J. (2000). In *Fatty Acids in Foods and Their Health Implications*, 2nd edn. (Chow, C.K., ed.), Marcel Dekker, Inc., New York, NY, pp. 153–174, with the exception of Atlantic Salmon that is adapted from Ackman, R.G. (2000). In *Fatty Acids in Foods and Their Health Implications*, 2nd edn. (Chow, C.K., ed.), Marcel Dekker, Inc., New York, NY, pp. 153–174.



X = OH = Phosphatidic acid

X = O—CH_2—CH_2—NH_2 = Phosphatidylethanolamine

X = O—CH_2—CH_2—$N^+(CH_3)_3$ = Phosphatidylcholine

X = O—CH_2—CH(NH_2)—COOH = Phosphatidylserine

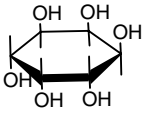
X =  = Phosphatidylinositol

FIGURE 4.3 Structures of phospholipids commonly found in foods.

fatty acids at *sn*-2 in milk fats may be a mechanism to insure that these fatty acids are absorbed by infant. Since long-chain saturated fatty acids at *sn*-1 and *sn*-3 are absorbed inefficiently, they provide less calories [13] and have less impact on blood lipid profiles. For example, when lard has its fatty acids randomly distributed and thus has more palmitic acid at *sn*-1 and *sn*-3, it increases plasma palmitic acid less than unmodified lard where 65% of palmitic acid is at *sn*-2. Structured triacylglycerols such as Salatrim have lower calories than normal fat because they have a high concentration of stearic acid (18:0) at *sn*-1 and *sn*-3 (see Section 4.8.2).

4.2.3 Phospholipids

The phospholipids or phosphoglycerides are modifications of triacylglycerols where phosphate groups are typically found in the *sn*-3 position (see Figure 4.3 for the structures of phospholipids). The simplest phospholipid is phosphatidic acid (PA) where the substitution group on the phosphate at *sn*-3 is an —OH. Other modifications of the substitution group on the phosphate at *sn*-3 result in phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (Figure 4.3). Nomenclature is similar to triacylglycerols with the name and location of the phosphate group coming at the end of the name (e.g., 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphoethanolamine). The term "lyso" signifies that a fatty acid has been removed from the phospholipid. In the food industry, lysophospholipids usually refer to a phospholipid where the fatty acid has been removed from the *sn*-2 position. Official nomenclature requires that the stereospecific location of the fatty acid removed should be named (e.g., 2-lysophospholipids, IUPAC). PC is commonly referred to as lecithin in the food industry, however, the lecithin sold as a food additive is not usually pure PC. Instead, it contains a mixture of a variety of different phospholipids, as well as some other components.

The presence of the highly polar phosphate group on phospholipids makes these compounds surface active. This surface activity allows phospholipids to arrange in bilayers that are critical for the properties of biological cell membranes. Since cell membranes need to maintain fluidity, the fatty acids found in phospholipids are often unsaturated to prevent crystallization at ambient temperatures. The fatty acids at the *sn*-2 position are typically more unsaturated than at the *sn*-1

position. The unsaturated fatty acids at the *sn*-2 position can be released by phospholipases so they can be utilized as substrates for enzymes such as cyclooxygenase and lipoxygenase (LOX). The surface activity of phospholipids means that they can be used to modify the physical properties of lipids by acting as emulsifiers and by modifying lipid crystallization behavior.

4.2.4 Sphingolipids

The sphingolipids are lipids that commonly contain a sphingosine base. Common sphingolipids include sphingomyelin (a sphingophospholipid; Figure 4.4), ceramides, cerebrosides, and gangliosides. These lipids are most commonly found associated with cell membranes especially in nervous tissue. They are generally not major components of food lipids.

4.2.5 Sterols

Sterols are derivatives of steroids. These nonpolar lipids all have three six-carbon rings and a five-carbon ring that is attached to an aliphatic chain (Figure 4.5). Sterols have a hydroxyl group attached to carbon 3 of the A ring. Sterol esters are sterols with a fatty acid esterified onto the hydroxyl group

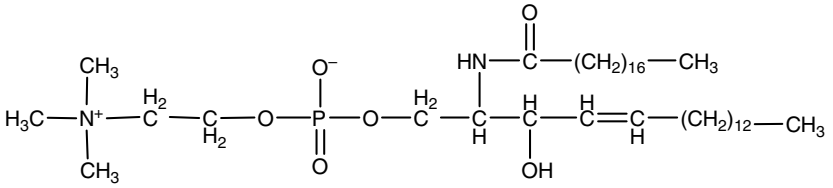


FIGURE 4.4 Structure of sphingomyelin, a common sphingolipid.

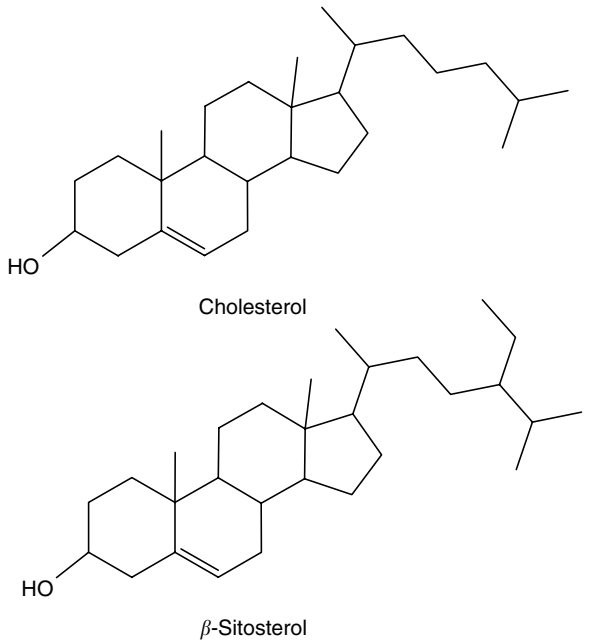


FIGURE 4.5 Structures of sterols commonly found in foods.

at carbon 3. Sterols are found in both plants (phytosterols) and animals (zoosterols). Cholesterol is the major sterol found in animal lipids. Plant lipids contain numerous sterols with β-sitosterol and stigmasterol predominating. Cholesterol can be found in plant lipids as a minor sterol component. The hydroxyl group at carbon 3 of sterols makes these compounds surface active. Cholesterol therefore can orient itself into cell membranes where it is important in stabilizing membrane structure. Cholesterol is also important because it is the precursor for the synthesis of bile acids and 7-dehydrocholesterol is the precursor for the production of vitamin D in the skin by ultraviolet (UV) irradiation [14]. High blood cholesterol and in particular high cholesterol in low-density lipoprotein (LDL) has been attributed to increased risk for cardiovascular disease. For this reason, reduced levels of dietary cholesterol are desirable. This can be achieved by reduction of animal fats in the diet and/or by removal of cholesterol from animal fats by supercritical carbon dioxide extraction or molecular distillation. Dietary phytosterols decrease cholesterol absorption in the intestine and therefore have been added to foods to reduce blood cholesterol levels (see Sections 4.8.1.1.1–4.8.1.1.4).

4.2.6 Waxes

The strict chemical definition of a wax is an ester of a long-chain acid and a long-chain alcohol. In reality, industrial and food waxes are a combination of chemical classes including wax esters, sterol esters, ketones, aldehydes, alcohols, hydrocarbons, and sterols [14]. Waxes can be classified according to their origin as animal (beeswax), plant (carnauba wax), and mineral (petroleum waxes). Waxes are found on the surface of plant and animal tissues to inhibit water loss or to repel water. Waxes are commonly added to the surface of fruits to slow dehydration during storage.

4.2.7 Miscellaneous Lipids

Other food lipids including the fat-soluble vitamins (A, D, E, and K) and carotenoids are covered in other sections of this book.

4.3 PHYSICOCHEMICAL PROPERTIES OF LIPIDS

This section will be primarily concerned with the physical properties of lipids and their influence on food properties. In particular, we will focus on how the molecular structure and organization of lipids determine their functional properties (e.g., melting characteristics, crystal morphology, and interactions) and how these functional properties determine the bulk physicochemical and sensory properties of food products (e.g., texture, stability, appearance, and flavor).

While there are a number of different categories of lipids present in food systems, this section will concentrate primarily on triacylglycerols because of their high natural abundance and major importance in food products. As mentioned earlier, triacylglycerols are esters of a glycerol molecule and three fatty acid molecules, and each fatty acid may have different numbers of carbon atoms, degrees of unsaturation, and branching (Section 4.2). The fact that there are many different types of fatty acids, and that these fatty acids can be located at different positions on the glycerol molecule, means that foods may contain a wide variety of different triacylglycerols. Indeed, edible fats and oils always contain a great many different types of triacylglycerol molecules or "species," with the precise type and concentration depending on their origin [15–17].

Triacylglycerol molecules have a "tuning-fork" structure, with the two fatty acids at the ends of the glycerol molecule pointing in one direction and the fatty acid in the *sn*-2 position pointing in the opposite direction (Figure 4.6). They are predominantly nonpolar molecules and so the most important types of molecular interaction that are responsible for their structural organization are van der Waals attraction and steric repulsion [18]. The interactions between two molecules can be described by the intermolecular pair potential $w(s)$, which is a measure of the strength of the attraction or repulsion between the molecules at a particular separation s (Figure 4.7). At a certain

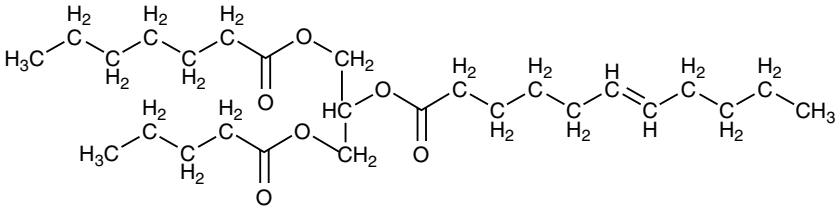


FIGURE 4.6 Chemical structure of a triacylglycerol molecule, which is assembled from three fatty acids and a glycerol molecule.

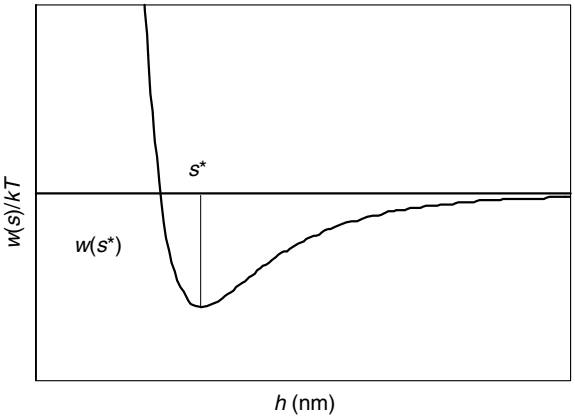


FIGURE 4.7 The strength of the attractive interactions between lipid molecules depends on the depth of the minimum in the overall molecular interaction potential.

molecular separation (s^*), there is a minimum in the intermolecular pair potential, which indicates that this is the most stable state. The value of s^* provides a measure of the average distance between triacylglycerols, while the depth of the pair potential at this value ($w(s^*)$) provides a measure of the strength of the attractive forces that hold the molecules together in the solid and liquid states (Figure 4.7). The structural organization of the molecules in triacylglycerols is primarily determined by their physical state, which depends on a balance between the attractive molecular interactions and the disorganizing influence of the thermal energy. Lipids exist as liquids above their melting point and as solids at temperatures that are sufficiently below their melting point to overcome supercooling effects (see below).

Lipid molecules may adopt a variety of different structural organizations in both the solid and liquid states depending on their precise molecular characteristics (e.g., chain length, degree of unsaturation, polarity) [19,20]. In the solid state, the organization of the lipid molecules may vary in several ways, including the overall organization of the triacylglycerol molecules relative to one another, the angle of tilt of the molecules within the crystal lattice, and the packing of the hydrocarbon chains. These differences mean that fat crystals can exist in a number of different polymorphic crystal forms (discussed later), which have different physical properties and melting behavior. Even in the liquid state, triacylglycerols are not randomly orientated but have some order owing to self-organization of the lipid molecules into structural entities (e.g., lamellar structures) [19,21]. The size and number of these structural entities is believed to decrease as the temperature is increased.

It should be noted that the term *fat* is conventionally used to refer to a lipid that is solid-like at room temperature, whereas the term *oil* is used to refer to a lipid that is liquid, although these terms are often used interchangeably [22,23].

TABLE 4.3
Comparison of Some Bulk Physicochemical Properties of a Liquid Oil (Triolein) and Water at 20°C

	Oil	Water
Molecular weight	885	18
Melting point (°C)	5	0
Density (kg m^{-3})	910	998
Compressibility (m s^2 kg^{-1})	5.03×10^{-10}	4.55×10^{-10}
Viscosity (mPa s)	≈50	1.002
Thermal conductivity (W m^{-1} K^{-1})	0.170	0.598
Specific heat capacity (J kg^{-1} K^{-1})	1980	4182
Thermal expansion coefficient ($°C^{-1}$)	7.1×10^{-4}	2.1×10^{-4}
Dielectric constant	3	80.2
Surface tension (mN m^{-1})	≈35	72.8
Refractive index	1.46	1.333

4.3.1 Physical Properties of Triacylglycerols

The physical properties of edible fats and oils depend primarily on the molecular structure, interactions, and organization of the triacylglycerol molecules that they contain [20,23–28]. In particular, the strength of the attractive interactions between the molecules and the effectiveness of their packing within a condensed phase largely determine their thermal behavior, density, and rheological properties (Table 4.3).

4.3.1.1 Rheological Properties

Most liquid oils are Newtonian liquids with intermediate viscosities, typically between 30 and 60 mPa s at room temperature [24,29]. Nevertheless, castor oil tends to have a much higher viscosity than most other oils because it contains an appreciable fraction of fatty acids with an alcohol group along their hydrocarbon backbones (i.e., ricinolenic acid), which is capable of forming relatively strong hydrogen bonds with neighboring molecules [24]. The viscosity of liquid oils tends to decrease steeply with increasing temperature and can be conveniently described by a logarithmic relationship [29].

Most "solid fats" actually consist of a mixture of fat crystals dispersed in a liquid oil matrix. The rheological properties of these solid fats are highly dependent on the concentration, morphology, interactions, and organization of the fat crystals present in the system [20,23]. Solid fats normally exhibit a type of rheological behavior known as "plasticity." A plastic material behaves like a solid below a critical applied stress, known as the yield stress (τ_0), but behaves like a liquid above this stress. The rheological behavior of an ideal plastic material, known as a *Bingham Plastic*, is shown in Figure 4.8. For an applied *shear* stress, the rheological characteristics of this type of material can be described by the following equation [23]:

$$\tau = G\gamma \quad (\text{for } \tau < \tau_0) \tag{4.1}$$

$$\tau - \tau_0 = \eta\dot{\gamma} \quad (\text{for } \tau \geq \tau_0) \tag{4.2}$$

where τ is the applied shear stress, γ is the resultant shear strain, $\dot{\gamma}$ is the rate of shear strain, G is the shear modulus (related to the strength or rigidity of material in response to shear strain), η is the

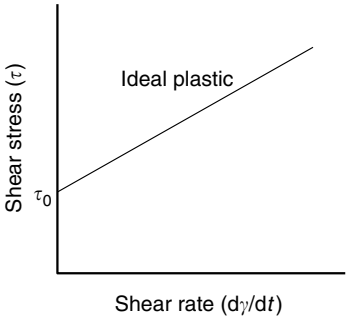


FIGURE 4.8 An ideal plastic material (*Bingham Plastic*) behaves like a solid below a critical applied stress, known as the yield stress (τ_0), but behaves like a liquid above this stress.

shear viscosity, and τ_0 is the yield stress (the point where the material starts to flow). In practice, solid fats tend to exhibit nonideal plastic behavior. For example, above the yield stress the fat may not flow like an ideal liquid and may exhibit non-Newtonian behavior (e.g., shear thinning). Below the yield stress, the fat might not behave as an ideal solid and exhibits some flow characteristics (e.g., viscoelasticity). In addition, the yield stress may not occur at a well-defined value, but may occur over a range of applied stresses because there is a gradual break down of the fat crystal network structure [30]. The yield stress of a fat tends to increase with increasing solid-fat content (SFC) and tends to be higher for crystal morphologies that are able to form three-dimensional networks that extend throughout the volume of the system more easily (i.e., small needle shaped crystals). A detailed discussion of the characteristics of plastic fats has recently been given elsewhere [23].

The structural origin of the plastic behavior of solid fats can be attributed to their ability to form a three-dimensional network of tiny fat crystals dispersed in a liquid oil matrix [23,31]. Below a certain applied stress there is a small deformation of the sample, but the weak bonds between the fat crystals are not disrupted. When the critical yield stress is exceeded, the weak bonds are broken and the fat crystals slide past one another leading to flow of the sample. Once the force is removed the flow stops, and the fat crystals begin to form bonds with their neighbors again. The rate at which this process occurs may have important implications for the functionality of the product. The influence of the rheological characteristics of triacylglycerols on the physicochemical and sensory properties of foods is described later.

4.3.1.2 Density

The density of a lipid is defined as the mass of material required to occupy a given volume [32]. This information is often important when designing food processing operations, since it determines the amount of material that can be stored in a tank or flow through a pipe of a given volume. The density of lipids is also important in certain food applications because it influences the overall properties of the system, for example, the creaming rate of oil droplets in oil-in-water (O/W) emulsions depends on the density difference between the oil and aqueous phases [33]. The densities of liquid oils tend to be around 910–930 kg m^{-3} at room temperature and tend to decrease with increasing temperature [24]. The densities of completely solidified fats tend to be around 1000–1060 kg m^{-3}, and they too decrease with increasing temperature [24]. In many foods, the fat is partially crystalline and so the density depends on the SFC, that is, the fraction of the total fat phase that is solidified. The density of a partially crystalline fat tends to increase as the SFC increases, for example, after cooling below the crystallization temperature. Measurements of the density of a partially crystalline fat can therefore sometimes be used to determine its SFC.

The density of a particular lipid depends primarily on the efficiency of the packing of the triacylglycerol molecules within it: the more efficient the packing, the higher the density.

Thus, triacylglycerols that contain linear saturated fatty acids are able to pack more efficiently than those that contain branched or unsaturated fatty acids, and so they tend to have higher densities [22,23]. The reason that solid fats tend to have higher densities than liquid oils is also because the molecules tend to be packed more efficiently. Nevertheless, this is not always the case [34]. For example, in lipid systems containing high concentrations of pure triacylglycerols that crystallize over a narrow temperature range, it has been shown that the density of the overall lipid system actually decreases on crystallization because of void formation.

Thermal properties: The most important thermal properties of lipids from a practical standpoint are the specific heat capacity (C_P), thermal conductivity (κ), melting point (T_{mp}), and enthalpy of fusion (ΔH_f) [24]. These thermal characteristics determine the total amount of heat that must be supplied (or removed) from a lipid system to change its temperature from one value to another, as well as the rate at which this process can be achieved. The specific heat capacities of most liquid oils and solid fats are around 2 J g^{-1}, and increase with increasing temperature [24]. Lipids are relatively poor conductors of heat and tend to have appreciably lower thermal conductivities ($\sim$0.165 W m^{-1} s^{-1}) than water ($\sim$0.595 W m^{-1} s^{-1}). Detailed information about the thermal properties of different kinds of liquid and solid lipids have been tabulated elsewhere [24,29]. Representative values are included in Table 4.3.

The melting point and heat of fusion of a lipid depend on the packing of the triacylglycerol molecules within the crystals formed: the more effective the packing, the higher the melting point and the enthalpy of fusion [18,23]. Thus, the melting points and heats of fusions of pure triacylglycerols tend to increase with increasing chain length. They are higher (1) for saturated fatty acids than for unsaturated fatty acids; (2) for straight-chained fatty acids than for branched fatty acids; (3) for triacylglycerols with a more symmetrical distribution of fatty acids on the glycerol molecule; (4) for *trans* than for *cis* unsaturated forms (Table 4.4); and (5) for more stable polymorphic forms (discussed later). The crystallization of lipids is one of the most important factors determining their influence on the bulk physicochemical and sensory properties of foods and therefore it will be treated in some detail in a later section.

TABLE 4.4
Melting Points and Heats of Fusion of the Most Stable Polymorphic Forms of Selected Triacylglycerol Molecules

Triacylglycerol	Melting Point (°C)	ΔH_f (J g^{-1})
LLL	46	186
MMM	58	197
PPP	66	205
SSS	73	212
OOO	5	113
LiLiLi	−13	85
LnLnLn	−24	—
SOS	43	194
SOO	23	—

L = lauric acid (C12:0), M = myristic acid (C14:0), P = palmitic acid (C16:0), S = stearic acid (C16:0), O = oleic acid (C18:1), Li = linoleic (C18:2), Ln = linolenic (C18:3).

Source: Adapted from Walstra, P. (2003). *Physical Chemistry of Foods*, Marcel Dekker, Inc., New York, NY.

For some applications, knowledge of the temperature where a lipid starts to breakdown owing to thermal degradation is important (e.g., frying or baking). The thermal stability of lipids can be characterized by their smoke, flash, and fire points [32]. The *smoke point* is the temperature at which the sample begins to smoke when tested under specified conditions. The *flash point* is the temperature at which the volatile products generated by the lipid are being produced at a rate where they can be temporarily ignited by application of a flame, but cannot sustain combustion. The *fire point* is the temperature at which the evolution of volatiles because of thermal decomposition occurs so quickly that continuous combustion can be sustained after application of a flame. Measurements of these temperatures are particularly important when selecting lipids that are going to be used at high temperatures (e.g., during baking or frying). The thermal stability of triacylglycerols is much better than that of free fatty acids, hence the propensity of lipids to breakdown during heating is largely determined by the amount of volatile organic material that they contain, such as free fatty acids [32].

Optical properties: Knowledge of the optical properties of lipids is important to food chemists for a number of reasons. First, the optical properties of lipids influence the overall appearance of many food materials [35]. Second, certain optical properties of lipids (e.g., refractive index and absorption spectrum) can be used to provide valuable information about their composition or quality [24,32]. The most important optical properties of lipids are their refractive index and absorption spectra. The refractive indices of liquid oils typically fall between 1.43 and 1.45 at room temperature [24]. The refractive index of a particular oil is mainly determined by the molecular structure of the fatty acids that it contains. The refractive index tends to increase with increasing chain length, increasing number of double bonds, and increasing conjugation of double bonds [24]. Empirical equations have been developed to relate the molecular structure of lipids to their refractive indices [24]. Hence, measurements of the refractive index of liquid oils can be used to provide some information about the average molecular weight or degree of unsaturation of the fatty acids that they contain. Measurements of the UV-visible absorption spectra of oils can also provide valuable information about their composition, quality, or molecular properties (e.g., presence of conjugated double bonds, carotenoids, or chlorophyll) [32]. For example, conjugated dienes adsorb UV light around 232 nm, whereas conjugated trienes adsorb around 270 nm.

The absorption spectrum of an oil can also have a pronounced influence on the final appearance of a food product. Pure triacylglycerols have little inherent color because they do not contain groups that adsorb light in the visible region of the electromagnetic spectrum. Nevertheless, commercial oils tend to be colored because they contain appreciable amounts of pigments that do absorb light (e.g., carotenoids and chlorophyll). For this reason, edible oils often undergo a decolorization step during their refinement. In emulsified foods, lipids also contribute to the opacity of the product because of their ability to scatter light, which is a direct result of the difference in refractive index between the lipid and aqueous phases.

Electrical properties: Knowledge of the electrical properties of lipids is sometimes important because several analytical techniques used to analyze fatty foods are based on measurements of their electrical characteristics, for example, electrical conductivity measurements of fat concentration or electrical pulse counting of fat droplet size [33]. Lipids tend to have fairly low relative dielectric constants ($\varepsilon_R \approx 2$–4) because of the low polarity of triacylglycerol molecules (Table 4.3). The dielectric constant of pure triacylglycerols tends to increase with increasing polarity (e.g., owing to the presence of $-$OH groups or owing to oxidation) and decreasing temperature [24]. Lipids also tend to be poor conductors of electricity, having relatively high electrical resistances.

4.3.2 Crystallization and Melting of Food Lipids

The physical state (solid or liquid) of the lipids in many food products plays an important role in their production and in determining their final quality attributes [20]. For example, the overall physicochemical and sensory properties of products such as margarine, butter, ice cream, whipped cream,

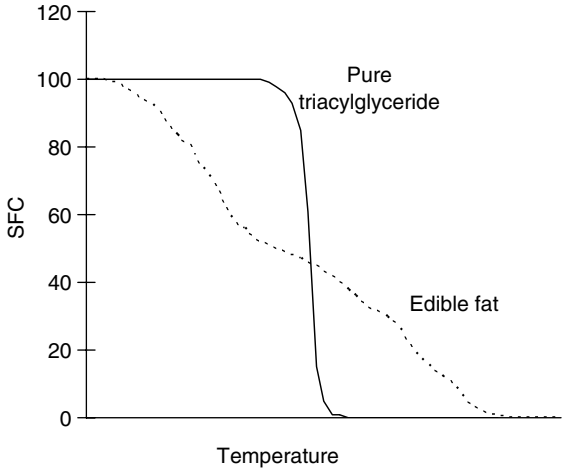


FIGURE 4.9 Comparison of the melting profile of a pure triacylglycerol and a typical edible fat. The edible fat melts over a much wider range of temperatures because it consists of a mixture of many different pure triacylglycerol molecules each with different melting points.

and baked goods are strongly influenced by the crystallization behavior of the lipids that they contain. The creation of food products with desirable properties therefore depends on an understanding of the major factors that influence the crystallization and melting of lipids in foods [19,23,26].

Solid fat content: The physical state of the lipids in a food is usually characterized in terms of the "SFC," which is the fraction (0–1) or percentage (0–100%) of lipid that is solid at a particular temperature. The temperature dependence of the SFC is one of the most important criteria influencing the selection of lipids for particular food applications, because it strongly influences the efficiency of the production process and the final properties of many fatty foods. The melting behavior of a pure triacylglycerol is shown schematically in Figure 4.9. The SFC falls from 100 to 0% when the temperature is increased from below to above the melting point (Figure 4.9). For a pure triacylglycerol the transition from solid-to-liquid occurs over a narrow range of temperatures close to the melting point (T_{mp}). The melting point of a pure triacylglycerol depends on the chain length, branching, and degree of unsaturation of its constituent fatty acids, as well as their relative positions along the glycerol molecule (Table 4.4). Edible fats contain a complex mixture of many different types of triacylglycerol molecules, each with a different melting point, and so they usually melt over a wide range of temperatures, rather than at a distinct temperature as would be the case for a pure triacylglycerol (Figure 4.9). As mentioned earlier, the desirable "plastic" rheological properties of edible fats usually occur over the range of temperatures where the lipids are partially crystalline (the "plastic range").

The melting profile of an edible fat is not simply the weighted sum of the melting profiles of its constituent triacylglycerols, because high melting point triacylglycerols are soluble in lower melting point ones [27]. For example, in a 50:50 mixture of tristearin and triolein it is possible to dissolve 10% of solid tristearin in liquid triolein at 60°C [22,28]. The solubility of a solid component in a liquid component can be predicted assuming they have widely differing melting points (>20°C):

$$\ln x = \frac{\Delta H_{fus}}{R}\left[\frac{1}{T_{mp}} - \frac{1}{T}\right] \tag{4.3}$$

Here x is the solubility, expressed as a mole fraction, of the higher melting point component in the lower melting point component, and ΔH_{fus} is the molar heat of fusion [22]. In addition, the melting

characteristics of food lipids depend on the nature of the fat crystals present (e.g., solid solution vs. mixed crystals, crystal morphology, and crystal polymorphic form [see later]).

The SFC of fatty foods is usually measured by calorimetry, changes in volume (dilatometry) or nuclear magnetic resonance (NMR). NMR is the preferred method to measure SFC since it requires little sample preparation and can be carried out quickly and simply [36]. SFC is an important parameter in food lipids because it provides information on important quality properties. Examples include crystallization behavior at refrigeration temperatures that will impact cloud point and emulsion stability, melting behavior at different temperatures that influences mouthfeel, baking properties, and spreadability of a lipid at refrigeration (tub margarine) or room temperature (stick margarine).

As mentioned earlier, the SFC-temperature profile of edible fats plays a major role in determining the functional and sensory properties of many fatty foods [16,22,27,28,37]. For example, it is important that margarines are "hard" enough to retain their shape when stored in a refrigerator or brought to room temperature, but that they are "soft" enough to be spread with a knife [38]. In addition, it is important that the fat crystals melt during mastication to provide a desirable mouthfeel. For this reason, it is important to use lipids that have SFC– and rheology–temperature profiles that are appropriate for specific applications [23].

4.3.3 Physicochemical Mechanism of Lipid-Phase Transitions

The arrangement of triacylglycerol molecules in the solid and liquid state is shown schematically in Figure 4.10. The physical state of a triacylglycerol at a particular temperature depends on its free energy, which is made up of contributions from both enthalpy and entropy terms: $\Delta G_{S\rightarrow L} = \Delta H_{S\rightarrow L} - T\Delta S_{S\rightarrow L}$ [39]. The enthalpy term ($\Delta H_{S\rightarrow L}$) represents the change in the overall strength of the molecular interactions between the triacylglycerols when they are converted from a solid to a liquid, whereas the entropy term ($\Delta S_{S\rightarrow L}$) represents the change in the organization of the molecules that is brought about by the melting process. The strength of the bonds between the lipid molecules is greater in the solid state than in the liquid state because the molecules are able to pack more efficiently, and so $\Delta H_{S\rightarrow L}$ is positive (unfavorable), which favors the solid state. On the other hand, the entropy of the lipid molecules in the liquid state is greater than that in the solid state, and therefore $\Delta S_{S\rightarrow L}$ is positive (favorable), which favors the liquid state. At low temperatures, the enthalpy term dominates the entropy term ($\Delta H_{S\rightarrow L} > T\Delta S_{S\rightarrow L}$), and therefore the solid state has the lowest free energy [19,23,39]. As the temperature increases, the entropy contribution becomes increasingly important. Above a certain temperature, known as the *melting point*, the entropy term dominates the enthalpy term ($T\Delta S_{S\rightarrow L} > \Delta H_{S\rightarrow L}$) and so the liquid state has the lowest free energy.

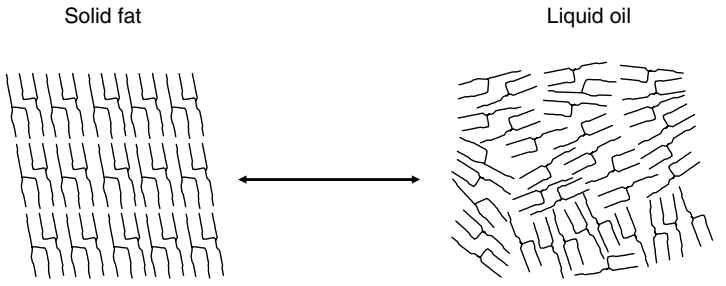


FIGURE 4.10 The arrangement of triacylglycerols in the solid and liquid states depends on a balance between the organizing influence of the attractive interactions between the molecules and the disorganizing influence of the thermal energy.

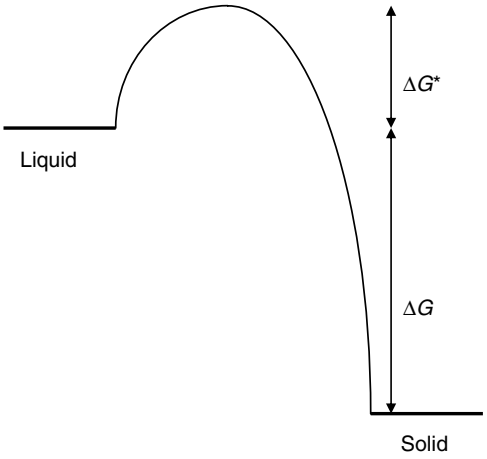


FIGURE 4.11 When the activation energy associated with nuclei formation is sufficiently high then a liquid oil can persist in a metastable state below the melting point of a fat.

A material therefore changes from a solid to a liquid when its temperature is raised above the melting point. A solid-to-liquid transition (melting) is endothermic because energy must be supplied to the system to pull the molecules further apart. Conversely, a liquid-to-solid transition (crystallization) is exothermic because energy is released as the molecules come closer together. Even though the free energy of the solid state is lowest below the melting point, solid crystals may not appear until a liquid oil has been cooled well below the melting point because of a free energy penalty associated with nuclei formation (see below).

Overall, the crystallization of fats can be conveniently divided into a number of stages: supercooling, nucleation, crystal growth, and postcrystallization events [19,20,23,40].

4.3.3.1 Supercooling

Although the solid form of a lipid is thermodynamically favorable at temperatures below its melting point, the lipid can persist in the liquid form below the melting point for a considerable period before any crystallization is observed. This is because of an activation energy associated with nuclei formation (ΔG^*) that must be overcome before the liquid–solid phase transition can occur (Figure 4.11). If the magnitude of this activation energy is sufficiently high compared to the thermal energy, crystallization will not occur on an observable timescale, and the system exists in a *metastable* state. The height of the activation energy depends on the ability of crystal nuclei to be formed in the liquid oil that are stable enough to grow into crystals (see below). The degree of supercooling of a liquid can be defined as $\Delta T = T - T_{mp}$, where T is the temperature and T_{mp} is the melting point. The value of ΔT at which crystallization is first observed depends on the chemical structure of the lipid, the presence of any contaminating materials, the cooling rate, the microstructure of the lipid phase (e.g., bulk vs. emulsified oil), and the application of external forces [19,23]. Pure oils containing no impurities can often be supercooled by more than 10°C before any crystallization is observed [41].

4.3.3.2 Nucleation

Crystal growth can only occur after stable nuclei have been formed in a liquid. These nuclei are believed to be clusters of oil molecules that form small-ordered crystallites, and are created when a number of lipid molecules collide and become associated with each other [19]. There is a free energy

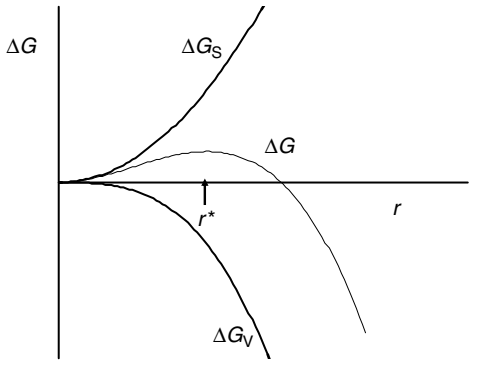


FIGURE 4.12 The critical size of a nucleus required for crystal growth depends on a balance between the volume and surface contributions to the free energy of nuclei formation. Nuclei that are spontaneously formed with radii below r^* grow, whereas those formed with radii below this value dissociate.

change associated with the formation of one of these nuclei (Figure 4.12). There is a negative free energy (ΔG_V) change that is proportional to the volume of the nucleus formed, which is because of the enthalpy and entropy changes that occur in the interior of the nucleus owing to the phase transition. On the other hand, the formation of a nucleus leads to the creation of a new interface between the solid and liquid phases, and this process involves an increase in free energy to overcome the interfacial tension. This positive free energy (ΔG_S) change is proportional to the surface area of the nucleus formed. The total free energy change associated with the formation of a nucleus is therefore a combination of a volume and a surface term [19,23]:

$$\Delta G = \Delta G_V + \Delta G_S = \frac{4}{3}\pi r^3 \frac{\Delta H_{fus}\Delta T}{T_{mp}} + 4\pi r^2 \gamma_i \tag{4.4}$$

where r is the radius of the nuclei, ΔH_{fus} is the enthalpy change per unit volume associated with the liquid–solid transition (which is negative), and γ_i is the solid–liquid interfacial tension. The volume contribution becomes increasingly negative as the size of the nuclei increases, whereas the surface contribution becomes increasingly positive (Figure 4.12). Since the surface area to volume ratio decreases with increasing size, the surface contribution tends to dominate for small nuclei, while the volume contribution tends to dominate for large nuclei. As a result, the overall free energy change associated with nuclei formation has a maximum value at a critical nucleus radius (r^*):

$$r^* = \frac{2\gamma_i T_{mp}}{\Delta H_{fus}\Delta T} \tag{4.5}$$

If a nucleus is spontaneously formed that has a radius that is below this critical size, then it will tend to dissociate so as to reduce the free energy of the system. On the other hand, if a nucleus is formed that has a radius that is above this critical value, then it will tend to grow into a crystal. This equation indicates that the critical size of nuclei required for crystal growth decreases as the degree of supercooling increases, which accounts for the increase in nucleation rate that is observed experimentally when the temperature is decreased. Practically, this means that liquid oils must be cooled appreciably below their thermodynamic melting points before crystal formation is observed.

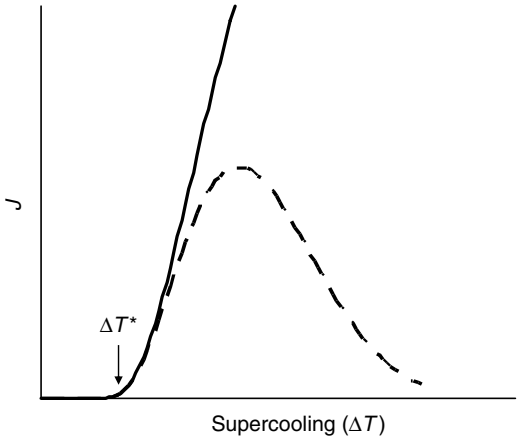


FIGURE 4.13 Theoretically, the rate of the formation of stable nuclei increases with supercooling (solid line), but in practice, the nucleation rate decreases below a particular temperature because the diffusion of oil molecules is retarded by the increase in oil viscosity (broken line).

The rate at which nucleation occurs can be mathematically related to the activation energy ΔG^* that must be overcome before stable nuclei are formed [19]:

$$J = A \exp(-\Delta G^*/kT) \tag{4.6}$$

where J is the nucleation rate, which is equal to the number of stable nuclei formed per second per unit volume of material, A is a preexponential factor, k is Boltzmann's constant, and T is the absolute temperature. The value of ΔG^* is calculated by replacing r in Equation 4.4 with the critical radius given in Equation 4.5. The variation of the nucleation rate predicted by Equation 4.5 with the degree of supercooling (ΔT) is shown in Figure 4.13. The formation of stable nuclei is negligibly slow at temperatures just below the melting point, but increases dramatically when the liquid is cooled below a certain temperature, T^*. In reality, the nucleation rate is observed to increase with the degree of cooling down to a certain temperature, after which it decreases on further cooling. This is because the increase in viscosity of the oil that occurs as the temperature is decreased slows down the diffusion of lipid molecules toward the liquid–nucleus interface [19,42]. Consequently, there is a maximum in the nucleation rate at a particular temperature (Figure 4.13).

The type of nucleation described above occurs when there are no impurities present in the oil, and is usually referred to as *homogeneous nucleation* [19]. If the liquid oil is in contact with foreign surfaces, such as the surfaces of dust particles, fat crystals, oil droplets, air bubbles, reverse micelles, or the vessel containing the oil, then nucleation can be induced at a higher temperature than expected for a pure system [19,23,43]. Nucleation owing to the presence of these foreign surfaces is referred to as *heterogeneous nucleation*, and can be divided into two types: primary and secondary. Primary heterogeneous nucleation occurs when the foreign surfaces have a different chemical structure to that of the oil, whereas secondary heterogeneous nucleation occurs when the foreign surfaces are crystals with the same chemical structure as the liquid oil. Secondary heterogeneous nucleation is the basis for "seeding" nucleation in supercooled lipids [19]. This process involves adding preformed triacylglycerol crystals to a supercooled liquid comprising the same triacylglycerol so as to promote nucleation at a higher temperature than would otherwise be possible.

Heterogeneous nucleation occurs when the impurities provide a surface where the formation of stable nuclei is more thermodynamically favorable than in the pure oil. As a result, the degree of supercooling required to initiate fat crystallization is reduced. On the other hand, certain types of impurities are capable of decreasing the nucleation rate of oils because they are incorporated into

the surface of the growing nuclei and prevent any further oil molecules from being incorporated [19]. Whether an impurity acts as a catalyst or an inhibitor of nucleation depends on its molecular structure and interactions with the nuclei [42,44]. It should be noted that there is still considerable debate about the mathematical modeling of nucleation, since existing theories often give predictions of nucleation rates that are greatly different from experimental measurements [23]. Nevertheless, the general form of the dependence of nucleation rates on temperature are predicted fairly well by existing theories (see Figure 4.13).

4.3.3.3 Crystal Growth

Once stable nuclei have formed they grow into crystals by incorporating molecules from the liquid oil at the solid–liquid interface [19,23,42]. Lipid crystals have a number of different faces, and each face may grow at an appreciably different rate, which partially accounts for the wide variety of different crystal morphologies that can be formed by food lipids. The overall crystal growth rate depends on several factors, including mass transfer of the molecules from the liquid phase to the solid–liquid interface, incorporation of the molecules within the crystal lattice, and removal of the heat generated by the crystallization process from the interface [19]. Environmental or system conditions such as viscosity, thermal conductivity, crystal structure, temperature profile, and mechanical agitation can influence the heat and mass transfer processes and therefore the rate of crystal growth. The crystal growth rate tends to increase initially with increasing degree of supercooling until it reaches a maximum rate, after which it decreases [19]. The dependence of the growth rate on temperature therefore shows a similar trend to the nucleation rate, however, the maximum rate of nuclei formation usually occurs at a different temperature to the maximum rate of crystal growth (Figure 4.14). This difference accounts for the dependence of the number and size of crystals produced on the cooling rate and holding temperature. If a liquid oil is cooled rapidly to a temperature where the nucleation rate is slower than the growth rate, then there will be a small number of large crystals formed. On the other hand, if a liquid oil is cooled to a temperature where the growth rate is slower than the nucleation rate, then there will be a large number of small crystals formed.

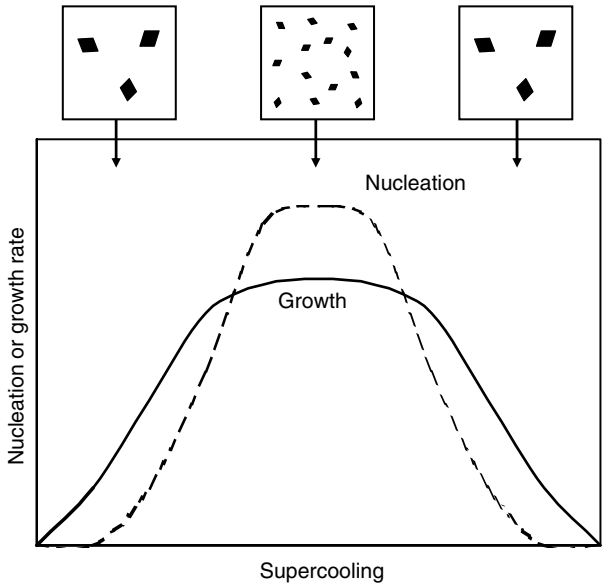


FIGURE 4.14 The nucleation and crystal growth rates have different temperature-dependencies, which account for differences in the number and size of fat crystals produced under different cooling regimes.

4.3.3.4 Postcrystallization Events

Once the crystals have been formed in a lipid system further changes in their packing, size, composition, and interactions can occur, even though the overall SFC may remain constant [19,23]. Postcrystallization may involve a change from a less stable to a more stable polymorphic form because of the rearrangement of the triacylglycerol molecules within the crystals. If a lipid forms mixed crystals (i.e., crystals that contain a mixture of different types of triacylglycerols), then there may be a change in the composition of the crystals during storage because of the diffusion of triacylglycerol molecules between the crystals. There may also be a net growth in the average size of the crystals within a lipid with time owing to Ostwald ripening, which is the growth of the large crystals at the expense of the smaller ones because of diffusion of lipid molecules between the crystals [19]. Finally, the bonds between fat crystals may strengthen over time during storage owing to a sintering mechanism (i.e., fusion of the crystals together) [23,27,28]. These postcrystallization changes can have pronounced influences on the bulk physicochemical and sensory properties of foods and therefore it is important to understand and control them. For example, postcrystallization events often lead to an increase in the size of the crystals in a lipid, which is undesirable in many cases because it leads to a gritty perception during consumption [43].

4.3.4 Crystal Structure

4.3.4.1 Morphology

The term "morphology" refers to the size, shape, and location of the crystals formed when a lipid crystallizes. The morphology of the crystals depends on a number of internal (e.g., molecular structure, composition, packing, and interactions) and external factors (e.g., temperature–time profile, mechanical agitation, and impurities). In general, when a liquid oil is cooled rapidly to a temperature well below its melting point a large number of small crystals are formed, but when it is cooled slowly to a temperature just below its melting point a smaller number of larger crystals are formed [19,23]. This is because of differences in the temperature dependence of the nucleation and crystallization rates (Figure 4.14). The nucleation rate tends to increase more rapidly with decreasing temperature than the crystallization rate up to a certain maximum value, and then it tends to decrease more rapidly with a further decrease in temperature. Thus, rapid cooling tends to produce many nuclei simultaneously that subsequently grow into small crystals, whereas slow cooling tends to produce a smaller number of nuclei that have time to grow into larger crystals before further nuclei are formed (Figure 4.14).

The structure and physical properties of crystals produced by cooling a complex mixture of triacylglycerols is also strongly influenced by the cooling rate and temperature [19,23,31]. If an oil is cooled rapidly, all the triacylglycerols crystallize at approximately the same time and a *solid solution* is formed, which consists of homogeneous crystals in which the triacylglycerols are intimately mixed with each other [22,23]. On the other hand, if the oil is cooled slowly, the higher melting point triacylglycerols crystallize first, while the low melting point triacylglycerols crystallize later, and so *mixed crystals* are formed. These crystals are heterogeneous and consist of some regions that are rich in high melting point triacylglycerols and other regions that are depleted in these triacylglycerols. Whether a fat forms mixed crystals or a solid solution influences many of its physicochemical properties, such as density, rheology, and melting profile [22,23], that could have an important influence on the properties of a food product.

4.3.4.2 Polymorphism

Triacylglycerols exhibit a phenomenon known as (monotropic) *polymorphism*, which is the ability of a material to exist in a number of crystalline structures with different molecular packing [19,23]. The three most commonly occurring types of packing in triacylglycerols are hexagonal,

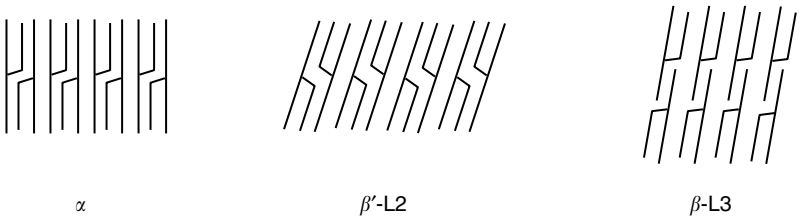


FIGURE 4.15 Common types of overall molecular organization of triacylglycerols within crystalline phases. (Adapted from Walstra, P. (2003). *Physical Chemistry of Foods*, Marcel Dekker, Inc., New York, NY.)

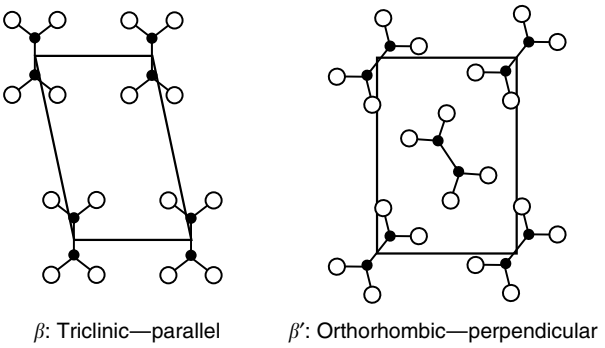


FIGURE 4.16 Two most common packing types of hydrocarbon chains: Triclinic (parallel) and orthorhombic (perpendicular). The black circles represent carbon atoms and the white circles represent hydrogen atoms. The hydrocarbon chains are viewed from the top. (Adapted from Larsson, K. (2004). In *Food Emulsions*, 4th edn. (Friberg, S., Larsson, K., and Sjoblom, J., eds.), Marcel Dekker, Inc., New York, NY, chap. 3.)

orthorhombic, and triclinic, which are usually designated as α, β', and β polymorphic forms, respectively (Figures 4.15 and 4.16). The type of crystalline form adopted depends on the molecular structure and composition of the lipids, as well as the environmental conditions during crystallization (cooling rate, holding temperature, shearing). The thermodynamic stability, and thus the melting point, of the three forms decreases in the order: $\beta > \beta' > \alpha$. Greater stability derives from greater packing density of fatty acyl groups and this is favored by homogeneity among the constitutive fatty acids and symmetry among the triacylglycerol species. Compatibility and segregation of fatty acids into the crystal lattice may give rise to unit cells with long spacings equivalent to either two or three fatty acid lengths in dimension (L2 and L3 in Figure 4.15). Even though the β form is the most thermodynamically stable, triacylglycerols often crystallize in the α form initially because it has the lowest activation energy for nuclei formation (Figure 4.17). With time the crystals transform to the most stable polymorphic form at a rate that depends on environmental conditions, such as temperature, pressure, and the presence of impurities [27]. The time taken for this type of crystal transformation to occur is strongly influenced by the homogeneity of the triacylglycerol composition [23]. The transition from the α form tends to occur fairly rapidly for relatively homogeneous compositions where the triacylglycerols all have fairly similar molecular structures. On the other hand, the transition is relatively slow for multicomponent fats where the triacylglycerols have diverse molecular structures. The different types of polymorphic forms of lipids can be distinguished from each other using a variety of methods, including x-ray diffraction, DSC, IR, NMR, and Raman spectroscopy [19]. These methods are largely based on the fact that the crystals are organized differently in different polymorphic forms that alter their physicochemical and structural properties (Figures 4.15, 4.16, and 4.18). Knowledge of the polymorphic form of the crystals in lipids is often important because it can

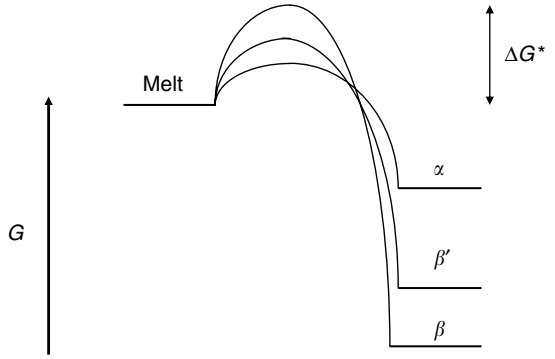


FIGURE 4.17 The polymorphic state that is initially formed when an oil crystallizes depends on the relative magnitude of the activation energies associated with nuclei formation.

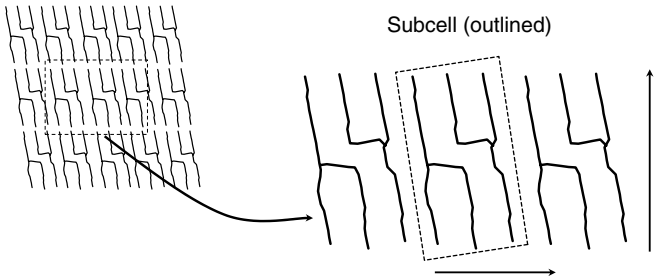


FIGURE 4.18 The unit cells in crystalline lipids can be characterized by their dimensions.

have a large impact on the thermal behavior and morphology of the crystals formed, and therefore on the physicochemical and sensory properties of foods. For example, the desirable textural characteristics and appearance of products such as margarine, spreads, baked goods, and chocolate depend on ensuring that the fat crystals are produced and maintained in the appropriate polymorphic form [19,38,43]. Finer β' crystals are preferred in margarines and spreads, where smoothness, gloss, and a high degree of surface coverage of dispersed water is required. Larger β polymorphic forms are often preferred in bakery shortening (e.g., lard) to create "flakiness" and cocoa butter stability in chocolate. Blending of lipids may also be used to control whether the β' or β polymorphs are the predominate crystal habits formed. Table 4.2 shows which edible lipids tend to form β' or β crystals as the most stable polymorph.

4.4 LIPID PROCESSING: ISOLATION, PURIFICATION, AND MODIFICATION

4.4.1 Lipid Refining

Triacylglycerols are extracted from both plant and animal sources. *Rendering* is a thermal processing operation that breaks down cellular structures to release the triacylglycerols from animal byproducts and underutilized fish species. Plant triacylglycerols can be isolated by pressing (olives) or solvent extraction (oilseeds) or a combination of the two (for detailed discussion of fat and oil extraction see Reference 45). The resulting crude oils and fats from these processes will not only contain triacylglycerols but also lipids (such as free fatty acids, phospholipids, lipid-soluble off-flavors, and carotenoids) as well as nonlipid materials (such as proteins and carbohydrates). These components

must be removed to produce oils and fats with the desired color, flavor, and shelf-life. The major refining steps are described below.

4.4.1.1 Degumming

The presence of phospholipids will cause the formation of water-in-oil (W/O) emulsions in fats and oils. These emulsions will make the oil cloudy and the water can present a hazard when the oils are heated to temperatures above 100°C (spattering and foaming). Degumming is a process that removes phospholipids by the addition of 1–3% water at 60–80°C for 30–60 min. Small amounts of acid are often added to the water to increase the hydrogen of the phospholipids in it. Settling, filtering, or centrifugation is then used to remove the coalesced "gums" formed by the phospholipids and water. With oils such as soybean, the phospholipids are recovered and sold as lecithin.

4.4.1.2 Neutralization

Free fatty acids must be removed from crude oils because they can cause off-flavors, accelerate lipid oxidation, cause foaming, and interfere with hydrogenation and interesterification operations. Neutralization is accomplished by mixing a solution of caustic soda with the crude oil, which causes the free fatty acids to form soluble soaps that can be removed by separating the oil phase from the water phase containing the soaps. The amount of caustic soda used is dependent on the free fatty acid concentrations in the crude oil. The resulting soap stock can be used as animal feed or to produce surfactants and detergents.

4.4.1.3 Bleaching

Crude oils often contain pigments that produce undesirable colors (carotenoids, gossypol, etc.) or promote lipid oxidation (chlorophyll). Pigments are removed by mixing the hot oil (80–110°C) with absorbents such as neutral clays, synthetic silicates, activated carbon, or activated earths. The absorbent is then removed by filtration. This process is usually done under vacuum since absorbents can accelerate lipid oxidation. An added benefit of bleaching is the removal of residual free fatty acids and phospholipids and the breakdown of lipid hydroperoxides.

4.4.1.4 Deodorization

Crude lipids contain undesirable aroma compounds such as aldehydes, ketones, and alcohols that occur naturally in the oil or are produced from lipid oxidation reactions that occur during extraction and refining. These volatile compounds are removed by subjecting the oil to steam distillation at high temperatures (180–270°C) and low pressures. Deodorization processes can also breakdown lipid hydroperoxides to increase the oxidative stability of the oil but may result in the formation of *trans* fatty acids. After deodorization is complete, citric acid (0.005–0.01%) is added to chelate and inactivate prooxidant metals. Deodorizer distillate will contain tocopherols and sterols that can be recovered and used as antioxidants and functional food ingredients (phytosterols).

4.4.2 Altering the SFC of Food Lipids

Natural fats with desirable plastic ranges are not always available and are sometimes expensive. In addition, alteration of fatty acid profiles is often desirable to make the fat less susceptible to oxidation (decrease unsaturation) or more nutritionally desirable (increase unsaturation). Therefore, several technologies have been developed to alter the SFC of food lipids.

4.4.2.1 Blending

The simplest method to alter fatty acid composition and melting profile is by blending fats with different triacylglycerol compositions. This practice is performed in products such as frying oils and margarines.

4.4.2.2 Dietary Interventions

The fatty acid composition of animal fats can be altered by manipulation of the type of fats in the diet. This practice is effective in nonruminants such as pigs, poultry, and fish. Increasing the levels of unsaturated fatty acids in fats from ruminants (cows and sheep) is not very efficient because bacteria in the rumen will biohydrogenate the fatty acids before they reach the small intestine where they can be absorbed into the blood.

4.4.2.3 Genetic Manipulation

The fatty acid composition of fats can be manipulated genetically by altering the enzyme pathways that produce unsaturated fatty acids. Genetic manipulation has been done successfully by both traditional breeding programs and by genetic modification technologies. Several oils that have been obtained from genetically altered plants such as sunflowers are commercially available. Most of these oils contain elevated levels of oleic acid.

4.4.2.4 Fractionation

The fatty acid and triacylglycerol composition of fats can also be altered by holding the fat at a temperature where the most saturated or long-chain triacylglycerols will crystallize and then collecting either the solid (more saturated or long-chain) or liquid (more unsaturated or short-chain) phases. This is commonly done to vegetable oils in a process called *winterization*. Winterization is necessary for oils used in products that are refrigerated to prevent the triacylglycerols from crystallizing and becoming cloudy. Winterization is also necessary for oils used in mayonnaise or salad dressings where crystallization would destabilize the emulsion.

4.4.2.5 Hydrogenation

Hydrogenation is a chemical process that adds hydrogen to double bonds. The process is used to alter lipids so they are more solid at room temperature, exhibit different crystallization behavior (by making the triacylglycerol composition more homogenous), and/or are more oxidatively stable. These goals are accomplished by the removal of double bonds to make the fatty acids more saturated. An additional use of hydrogenation is to bleach oils since the destruction of double bonds in compounds such as carotenoids will cause them to lose color. Products produced by hydrogenation include margarines, shortenings, and partially hydrogenated oils that have improved oxidative stability.

The hydrogenation reaction requires a catalyst to speed up the reaction, hydrogen gas to provide the substrate, temperature control to initially heat up the oil to make it liquid and then cool the oil once the exothermic reaction is started, and agitation to mix the catalyst and substrates [45]. The oil used in hydrogenation must first be refined since contaminants will reduce the effectiveness of, or "poison," the catalysts. Hydrogenation is done as a batch or continuous process at temperatures ranging from 250°C to 300°C. Reduced nickel is the most common catalyst that is added at 0.01–0.02%. The nickel is incorporated onto a porous support to provide a catalyst with high surface area that can be recovered by filtration. Continuous mixing is a critical parameter since mass transfer of the reactants limits the reaction. The reaction takes 40–60 min during which progress is monitored by change in

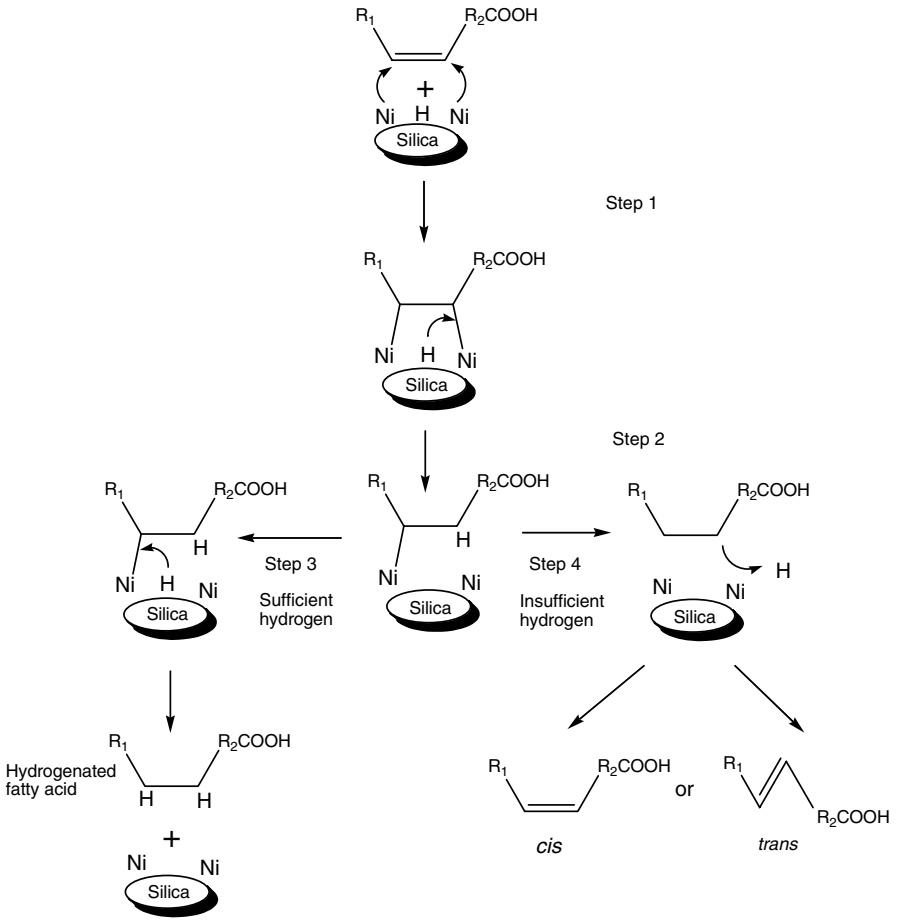


FIGURE 4.19 The pathways involved in hydrogenation that led to formation of saturated fatty acids and *cis* and *trans* unsaturated fatty acids.

refractive index. Upon completion, catalysts are recovered by filtration so that they can be used in another reaction.

The mechanism of hydrogenation involves initial complexation of the unsaturated fatty acid with the catalyst at each end of the double bond (Figure 4.19, step 1). Hydrogen that is absorbed to the catalysts can then break one of the carbon–metal complexes to form a half-hydrogenated state with the other carbon remaining linked to the catalyst (step 2). To complete hydrogenation, the half-hydrogenated state interacts with another hydrogen to break the remaining carbon–catalyst bond to produce a hydrogenated fatty acid (step 3). However, if hydrogen is not available, the reverse reaction can occur and the fatty acid is released from the catalyst and the double bond reforms (step 4). The double bond that reforms can be in the *cis* or *trans* configuration (geometric isomers) and can be at the same carbon number or it can migrate to the adjacent carbon (e.g., a fatty acid with a double bond originally between carbons 9 and 10 can migrate to carbons 8 and 9 or 10 and 11; positional isomers). The propensity of the double bond to reform is related to the concentration of hydrogen associated with the catalyst. Thus, conditions such as low hydrogen pressure, low agitation, high temperature (reaction is faster than rate of hydrogen diffusion to the catalyst), and high catalyst concentrations (difficult to saturate catalyst with hydrogen) result in high levels of geometric and positional isomers. This can be problematic since dietary *trans* fatty acids is associated with increased cardiovascular disease risk.

Hydrogenation often proceeds in a selective and sequential manner. The rate of hydrogenation of polyunsaturated fatty acids is faster than monounsaturated fatty acids. This is partially due to the higher catalyst affinity for pentadiene double bond systems in polyunsaturated fatty acids than for monounsaturated fatty acids. Preferential hydrogenation of the most unsaturated fatty acid is especially prevalent when hydrogen concentration at the catalyst is low. From a stability standpoint, hydrogenation of the most unsaturated fatty acids first is often desirable since this increases the oxidative stability of the oil with minimal formation of high temperature melting saturated triacylglycerols that cause problems with crystallization and texture. However, low hydrogen concentrations can also lead to high production of geometric and positional isomers meaning that the lipid can contain high amounts of *trans* fatty acids, which is nutritionally undesirable.

4.4.2.6 Interesterification

Interesterification is a process that involves the rearranging of acyl groups in triacylglycerols. Generally, this is a random process that results in production of a triacylglycerol profile different from that of the original lipid. This results in significant changes in the melting profiles of lipids without changing fatty acid composition [46]. Interesterification also alters crystallization behavior of the fat by making it more difficult for the lipids to form the most stable crystal type (β, triclinic) since the triacylglycerol composition becomes more heterogeneous. Interesterification is performed by acidolysis, alcoholysis, glycerolysis, and transesterification [46]. Transesterification is the most common method used to alter the properties of food lipids. In this process, alkylates of sodium (e.g., sodium ethylate) are commonly used to accelerate transesterification since they are inexpensive and active at low temperatures. The real catalyst for the reaction is thought to be a carbonyl anion of a diacylglycerol (Figure 4.20). The negative diacylglycerol can attack the slightly positive carbonyl group of a fatty acid on a triacylglycerol to form a transition complex. Upon transesterification the transition complex decomposes in such a way that the fatty acid is transferred to the diacylglycerols and the anion migrates to the site of the transferred fatty acid. The transesterification process can occur within the same (intraesterification) or a different (interesterification) triacylglycerol. For interesterification to take place the reaction medium must have, low levels of water, free fatty acids, and peroxides (that deactivate the catalyst). Random transesterification is performed at 100–150°C and is complete in 30–60 min. The reaction is stopped by the addition of water to inactivate the catalyst.

Interesterification can be performed on mixtures of lipids such as a fat with a high temperature melting range and an oil with a low temperature melting range. If these two lipid sources were simply blended, their melting profile could have a discontinuous, stair-stepped SFC curve (cf. Figure 4.9) as the blend is progressively heated. Interesterification of these two lipids would create new triacylglycerols containing combinations of both saturated and unsaturated fatty acids and having gradual melting throughout the plastic range. Another application would be to interesterify a fat with a very homogeneous triacylglycerol composition to produce heterogeneous triacylglycerols; this would widen the plastic range and prompt β' (orthorhombic) crystals as the most stable polymorphs.

Interesterification is not always random. In directed interesterification, the reaction temperature is held low enough so that when highly saturated triacylglycerols are produced, they crystallize and are removed from participation in the reaction. This process would produce a liquid phase that is more unsaturated and a solid phase that is more saturated than the parent lipid. Interesterification can also be performed by using lipases as catalysts [47]. The advantage of lipases is that they can have specificity for different stereospecific locations on the triacylglycerol or specificity for different fatty acids. This means that structured triacylglycerols can be produced with changes in fatty acid composition or triacylglycerol type (e.g., changes at *sn*-2 position). By altering fatty acid and/or triacylglycerol composition these fats may have superior nutritional or physical properties. Unfortunately, enzymatic interesterification is limited by its high cost and its application is limited to products of high value such as cocoa butter substitutes and infant formula lipids.

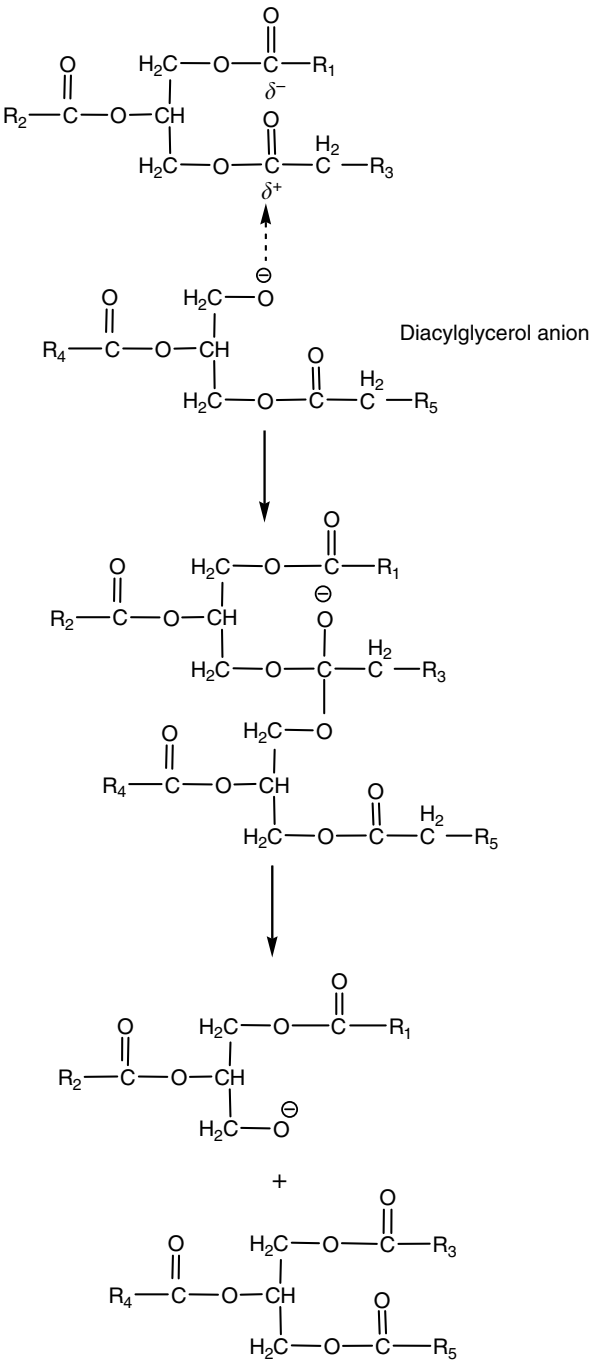


FIGURE 4.20 The proposed mechanism of the interesterification reaction involving catalysis by the carbonyl anion of a diacylglycerol. (Adapted from Rousseau, D. and Marangoni, A.G. (2002). In *Food Lipids, Chemistry, Nutrition and Biotechnology* (Akoh, C.C. and Min, D.B., eds.), Marcel Dekker, Inc., New York, NY, pp. 301–334.)

4.5 FUNCTIONALITY OF TRIACYLGLYCEROLS IN FOODS

The ability of food scientists to improve the quality of food products depends on an improved understanding of the multiple roles that fats and oils play in determining their properties. This section highlights some of the most important roles that lipids play in determining the texture, appearance, and flavor of food products, using specific examples to highlight important aspects of triacylglycerol functionality.

4.5.1 Texture

The influence of lipids on the texture of foods is largely determined by the physical state of the lipid and the nature of the food matrix (e.g., bulk fat, emulsified fat, or structural fat). For bulk liquid oils, such as cooking or salad oils, the texture is determined primarily by the viscosity of the oil over the temperature range of utilization. For partially crystalline fats, such as in chocolate, baked products, shortenings, butter, and margarine, the texture is mainly determined by the concentration, morphology, and interactions of the fat crystals [19,23,43]. In particular, the melting profile of the fat crystals plays a major role in determining properties such as texture, stability, spreadability, and mouthfeel. The characteristic creamy texture of many O/W food emulsions is determined by the presence of fat droplets (e.g., creams, desserts, dressings, and mayonnaise). In these systems, the viscosity of the overall system is determined mainly by the concentration of oil droplets present rather than by the viscosity of the oil within the droplets [33]. For example, whole milk (~4% fat) has a relatively low viscosity, heavy cream (~40% fat) is highly viscous, and mayonnaise (~80% fat) is semisolid, even though the viscosity of the oil phase within the droplets may be fairly similar among these products. In W/O food emulsions, the overall rheology of the system is largely determined by the rheology of the oil phase. In most food W/O emulsions, such as margarine, butter, and spreads, the oil phase is partially crystalline and has plastic-like properties. The rheology of these products is therefore determined by the SFC and the morphology and interactions of the fat crystals present, which in turn is governed by the crystallization and storage conditions (see below). For example, the "spreadability" of these products is determined by the formation of a three-dimensional network of aggregated fat crystals in the continuous phase that provides the product with mechanical rigidity [31,48]. In many foods, the lipids form an integral part of a solid matrix that also contains various other components (e.g., chocolate, cakes, cookies, biscuits, cheese). The physical state of the lipids in these systems impacts their texture by forming a network of interacting fat crystals that gives the final product desirable rheological properties, such as firmness or snap. The presence of the fat phase in these products may also influence the overall texture in a variety of other ways and a few examples of food products where lipids play a major role on their texture are given below.

Margarine production is a good example of the importance of lipid crystallization on determining the overall texture of food products. Initially, the manufacturer must select a lipid phase that contains a blend of triacylglycerols that will provide the appropriate SFC–temperature profile and crystal morphology in the final product. This lipid phase is then homogenized in its liquid state with an aqueous phase to form an O/W emulsion. This emulsion is then processed under carefully controlled time–temperature–shear conditions to obtain the desired extent of crystallization, crystal size, polymorphic form, and degree of crystal interaction [38]. Ideally, the final product should contain a three-dimensional network of small aggregated crystals in the β' polymorphic form, as this provides the desired textural and stability characteristics. Margarine production is usually carried out by passing the O/W emulsion through a "scraped surface heat exchanger" followed by a "crystallizer." In the scraped surface heat exchanger the emulsion is rapidly cooled and exposed to high shear rates, which promotes the rapid formation of fat crystals in the lipid phase. The presence of fat crystals in the oil droplets promotes the conversion of the O/W emulsion into a W/O emulsion owing to partial coalescence [23]. The W/O emulsion consists of water droplets embedded in a lipid

phase that contains a network of aggregated crystals. The fat crystals formed are initially in the α polymorphic form but are converted to the more stable β' polymorphic form during the crystallization step. It is important to control the extent of this transformation during the manufacturing process since this determines the number and strength of the bonds formed between the fat crystals and therefore the rheology of the final product. On the other hand, it is also important to prevent the polymorphic transition from the β' form to the more stable β form during storage, since this leads to the formation of large crystals (>30 μm) that are perceived as "grainy" or "gritty" in the mouth. This conversion can often be prevented by adding surfactants that interfere with the polymorphic transition [1], choosing lipids that will not form β crystals, or by appropriate blending of lipids to favor the β' crystal habit.

Another example of the importance of fat crystallization on the texture of food products is provided by shortenings. Shortenings are fats that are used to provide characteristic functional properties to a variety of different food products, including cakes, breads, pastry, fried products, and baked products [43]. These functional properties include tenderness, texture, mouthfeel, structural integrity, lubrication, incorporation of air, heat transfer, and extended shelf-life. Various physicochemical mechanisms underlie these functional properties. Shortenings are named as such because they help to prevent interactions between proteins or starch molecules, which serve to "tenderize" the product by reducing gluten cohesion and "shortening" the texture [43]. They provide textural characteristics in other foods because of their ability to form a three-dimensional fat crystal network. To obtain the desired functional characteristics in a particular product, it is important to choose a blend of fats and oils that gives the appropriate melting profile and polymorphic characteristics, then to process the fat using controlled cooling and shearing conditions to obtain the desired crystal type and structure [43]. It is usually important that the lipid is partially crystalline at storage temperatures so that it maintains its structural integrity, but melts during consumption to give a desirable mouthfeel.

4.5.2 Appearance

The characteristic appearance of many food products is strongly influenced by the presence of lipids. The color of bulk oils, such as cooking or salad oils, is mainly determined by the presence of pigment impurities that adsorb light, such as chlorophyll and carotenoids. Solid fats are usually optically opaque because of scattering of light by the fat crystals present, whereas liquid oils are usually optically clear. The opacity of the fat depends on the concentration, size, and shape of the fat crystals present. The turbid, cloudy, or opaque appearance of food emulsions is a direct result of the immiscibility of oil and water, since this leads to a system where the droplets of one phase are dispersed in the other phase. Food emulsions usually appear optically opaque because the light passing through them is scattered by the droplets [49]. The intensity of the scattering depends on the concentration, size, and refractive index of the droplets present, so that both the color and opacity of food emulsions are strongly influenced by the presence of the lipid phase. The reason that whole milk ($\sim$4% fat) has a much whiter appearance that skim milk ($<$0.1% fat) is because it contains milk fat globules that scatter light strongly, whereas skim milk only contains casein micelles that scatter light more weakly.

An interesting example of the importance of fat crystallization on the appearance of food products is "bloom," which is a quality defect sometimes observed in chocolates and coatings [50]. Bloom manifests itself as large white spots or a dull-whitish gray appearance on the surface of the product. A variety of mechanisms have been proposed to account for bloom in different products, all of which are due to some stability problem associated with fat crystallization (e.g., poor tempering, incompatibility of fat blends, fat migration, and fat recrystallization). The physicochemical basis of the various mechanisms have been discussed in detail elsewhere [50]. Bloom is a defect that occurs in some chocolate products that have been exposed to fluctuating temperatures during their storage, since this causes the fat phase to melt and recrystallize [1]. It has been proposed that the fat crystals

at the surface change their morphology from flat smooth crystals that specularly reflect light and give a smooth appearance to spiky crystals that diffusely reflect light and cause a cloudy appearance. Bloom can often be retarded or prevented by using surfactants that limit the crystal transition or by carefully controlling the storage temperature to avoid fat polymorphic phase transitions [1].

4.5.3 Flavor

Triacylglycerols are relatively large molecules that have a low volatility and hence little inherent flavor. Nevertheless, edible fats and oils from different natural sources have distinctive flavor profiles because of the presence of characteristic volatile compounds, such as lipid oxidation products and natural impurities. Minor fatty acid constituents can also contribute subtle flavor notes, especially in animal fats (Chapter 10). The flavor of many food products is indirectly influenced by the lipid phase because flavor compounds can partition between oil, water, and gaseous regions within the food matrix according to their polarities and volatilities [33]. For this reason, the perceived aroma and taste of foods are often strongly influenced by the type and concentration of lipids present.

Lipids also influence the mouthfeel of many food products [23]. Liquid oils may coat the tongue during mastication, which provides a characteristic oily mouthfeel. Fat crystals also confer a "grainy" or "gritty" mouthfeel if they are large, and "smoothness in texture" if they are small [38]. The melting of fat crystals in the mouth causes a cooling sensation, which is an important sensory attribute of many fatty foods [22].

4.6 CHEMICAL DETERIORATION OF LIPIDS: HYDROLYTIC REACTIONS

Free fatty acids cause problems in foods because they produce off-flavor, reduce oxidative stability, cause foaming, and reduce smoke point (the temperature at which an oil begins to smoke). If the liberation of free fatty acids from the glycerol backbone results in the development of off-flavors (e.g., volatile short-chain free fatty acids that form off-aromas or long-chain fatty acids that form soapy tastes), this is known as *hydrolytic rancidity*. However, short-chain free fatty acids are sometimes desirable in products, such as cheeses, where they contribute to flavor profiles.

Free fatty acids can be liberated from triacylglycerols by enzymes called lipases. In living tissues, the activity of (phospho)lipases is strictly controlled since fatty acids can be cytotoxic by disrupting cellular membrane integrity. During processing and storage of the biological tissues used as raw materials for foods, cellular structures and biochemical control mechanisms may be destroyed and lipases can become active (e.g., can come in contact with lipid substrates). A good example of this is seen in the production of olive oil where the oil from the first pressing has low free fatty acid concentrations. Oils from subsequent pressing and oil extracted from the pomace have higher free fatty acid concentrations as the cellular matrix is further disrupted and the lipases have time to hydrolyze triacylglycerols. Triacylglycerol hydrolysis also occurs in frying oils owing to the high processing temperatures and the introduction of water from the fried food. As the free fatty acid content of the frying oil increases, smoke point and oxidative stability decrease and the tendency for foaming increases. Commercial frying oils are filtered on a regular basis with absorbents that are capable of binding and removing free fatty acids to increase the shelf-life of the oil. Triacylglycerol hydrolysis will also occur at extreme pH values.

4.7 CHEMICAL DETERIORATION OF LIPIDS: OXIDATIVE REACTIONS

"Lipid oxidation" is a general term that is used to describe a complex sequence of chemical changes that result from the interaction of lipids with oxygen [51,52]. Triacylglycerols and phospholipids

have low volatility and thus do not directly contribute to the aroma of foods. During lipid oxidation reactions, the fatty acids esterified to triacylglycerols and phospholipids will decompose to form small, volatile molecules that produce the off-aromas known as *oxidative rancidity*. In general, these volatile compounds are detrimental to food quality although there are some food products, such as fried foods, dried cereal, and cheeses, for which small amounts of lipid oxidation products are important positive components for their flavor profile.

4.7.1 Mechanisms of Lipid Oxidation

The centerpiece of these reactions is the molecular species known as free radicals. Free radicals are molecules or atoms that have unpaired electrons. Free radical species can vary greatly in their energy. Radicals such as the hydroxyl radical ($^{\bullet}OH$) have very high energy and can oxidize virtually any molecule by causing hydrogen abstraction. Other molecules such as the antioxidant, α-tocopherol, can form free radicals with low energy that are less capable of attacking molecules such as unsaturated fatty acids.

The kinetics of lipid oxidation in foods often has a lag phase followed by an exponential increase in oxidation rate (Figure 4.21). The length of the lag phase is very important to food processors since this is the period where rancidity is not detected and the quality of the food is high. Once the exponential phase is reached, lipid oxidation proceeds quickly and off-aroma development occurs rapidly. The length of the lag phase of oxidation will increase with decreasing temperature, oxygen concentrations, degree of fatty acid unsaturation, activity of prooxidants, and increasing concentrations of antioxidants. Figure 4.21 shows how gamma-tocopherol can increase the lag phase of the oxidation of a corn O/W emulsion [53].

Oxidation can occur in both free fatty acids and fatty acyl groups. The pathway of fatty acid oxidation can be described by three general steps: initiation, propagation, and termination.

Initiation: This step describes the abstraction of a hydrogen from a fatty acid to form a fatty acid radical known as the alkyl radical ($L^{\bullet}$). Once the alkyl radical forms, the free radical is stabilized by delocalization over the double bond(s) resulting in double bond shifting, and in the case of polyunsaturated fatty acids by the formation of conjugated double bonds. This shift in location can produce double bonds in either the *cis* or *trans* configuration with *trans* predominating because of their greater stability. Figure 4.22 shows the initiation steps for hydrogen abstraction from the

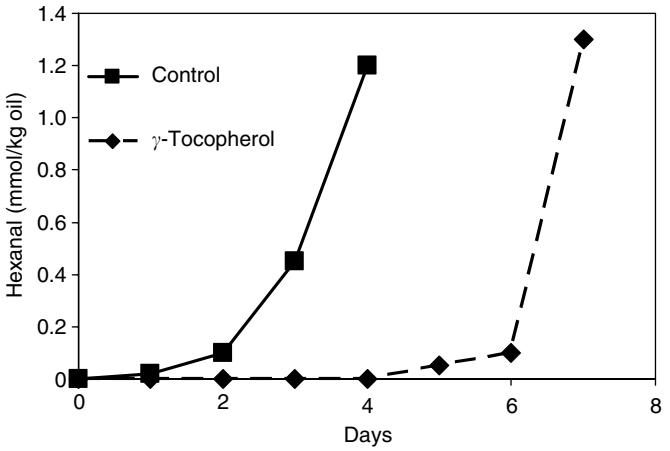


FIGURE 4.21 The impact of γ-tocopherol on the lag phase of the oxidation of a corn O/W emulsion. (Adapted from Huang, S.W., Frankel, E.N., and German, J.B. (1994). *J. Agric. Food Chem.* 42: 2108–2114.)

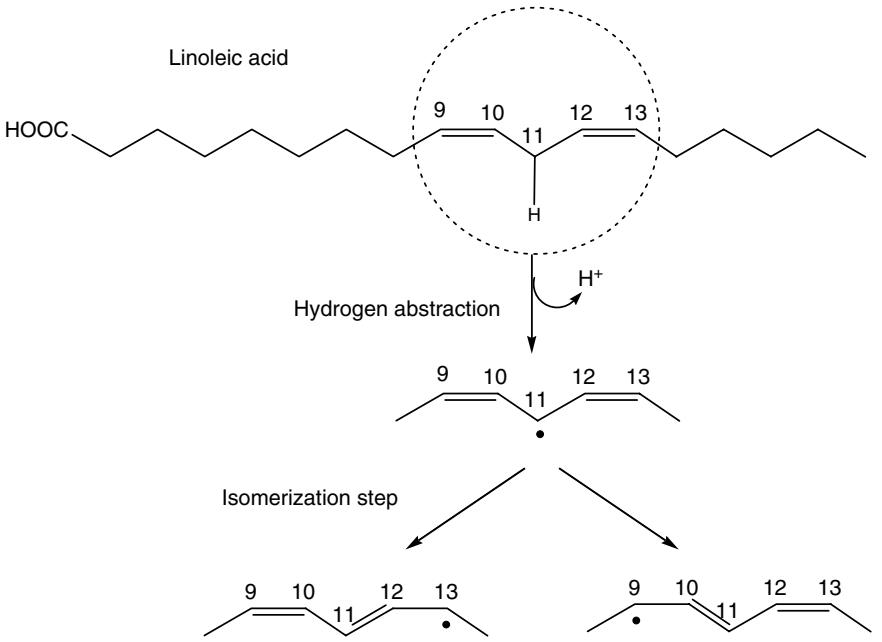


FIGURE 4.22 The initiation step of lipid oxidation for linoleic acid.

methylene-interrupted carbon of linoleic acid with double bond rearrangement producing two isomers. When hydrogen is abstracted from oleic acid, the alkyl radical can exist at four different locations (Figure 4.23).

The ease of formation of fatty acid radicals increases with increasing unsaturation. The bond dissociation energy for the carbon–hydrogen covalent bond in an aliphatic chain is 98 kcal mol^{-1}. If a carbon atom is adjacent to an electron-rich double bond, the carbon–hydrogen covalent bond becomes weaker with the bond dissociation energy decreasing to 89 kcal mol^{-1}. In polyunsaturated fatty acids, the double bonds are in a pentadiene configuration with a methylene-interrupted carbon (Figure 4.24). Since the carbon–hydrogen covalent bond of the methylene-interrupted carbon is weakened by two double bonds, its bond dissociation energy is even lower at 80 kcal mol^{-1}. As the bond dissociation energy of the carbon–hydrogen bond decreases, hydrogen abstraction becomes easier and lipid oxidation is faster. Linoleic acid (18:2) has been estimated to be 10–40 times more susceptible to oxidation than oleic acid (18:1). As additional double bonds are added onto polyunsaturated fatty acids, an additional methylene-interrupted carbon is added producing another site for hydrogen abstraction. For example, linoleic (18:2) has one methylene-interrupted carbon while linolenic (18:3) has two and arachidonic (20:4) has three (Figure 4.24). In most cases, oxidation rates double with the addition of a methylene-interrupted carbon. Thus, linolenic oxidizes twice as fast as linoleic and arachidonic oxidizes twice as fast as linolenic (four times faster than linoleic).

Propagation: The first step of propagation involves the addition of oxygen to the alkyl radical. Atmospheric or triplet oxygen is a biradical because it contains two electrons with the same spin direction that cannot exist in the same spin orbital. The free radicals on triplet oxygen are low energy and will not directly cause hydrogen abstraction. However, the free radicals on oxygen can react with the alkyl radical at a diffusion-limited rate. The combination of the alkyl radical with one of the radicals on triplet oxygen results in the formation of a covalent bond. The other radical on the oxygen remains free. The resulting radical is known as a peroxyl radical ($LOO^{\bullet}$). The high energy of peroxyl radicals allows them to promote the abstraction of a hydrogen from another molecule. Since the carbon–hydrogen covalent bond of unsaturated fatty acids is weak, they are susceptible

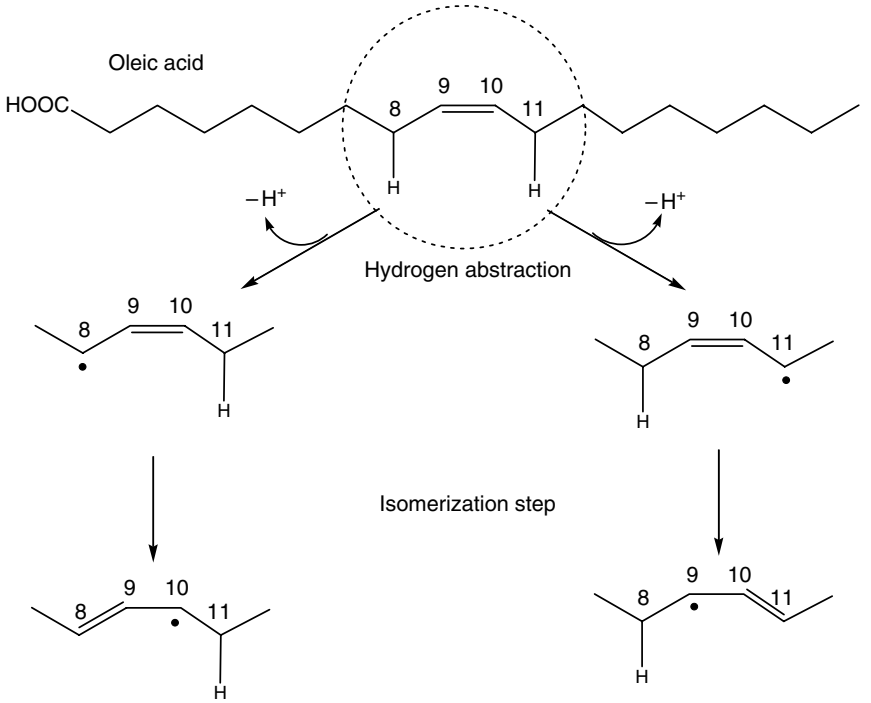


FIGURE 4.23 The initiation step of lipid oxidation for oleic acid.

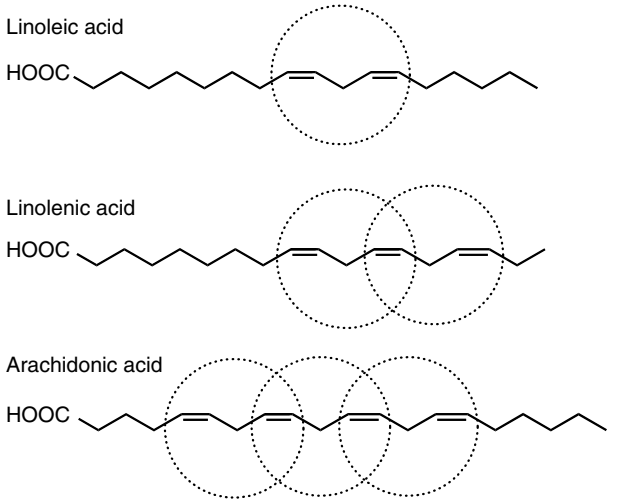


FIGURE 4.24 The pentadienes of linoleic, linolenic, and arachidonic acids.

to attack from peroxyl radicals. Hydrogen addition to the peroxyl radical results in the formation of a fatty acid hydroperoxide (LOOH) and the formation of a new alkyl radical on another fatty acid. Thus the reaction is propagated from one fatty acid to another. A schematic of this pathway for two linoleic molecules is shown in Figure 4.25. The location of the lipid hydroperoxide will correspond to the location of alkyl radicals (shown in Figures 4.22 and 4.23). Thus, oleate will produce four hydroperoxides, and linoleate will form two.

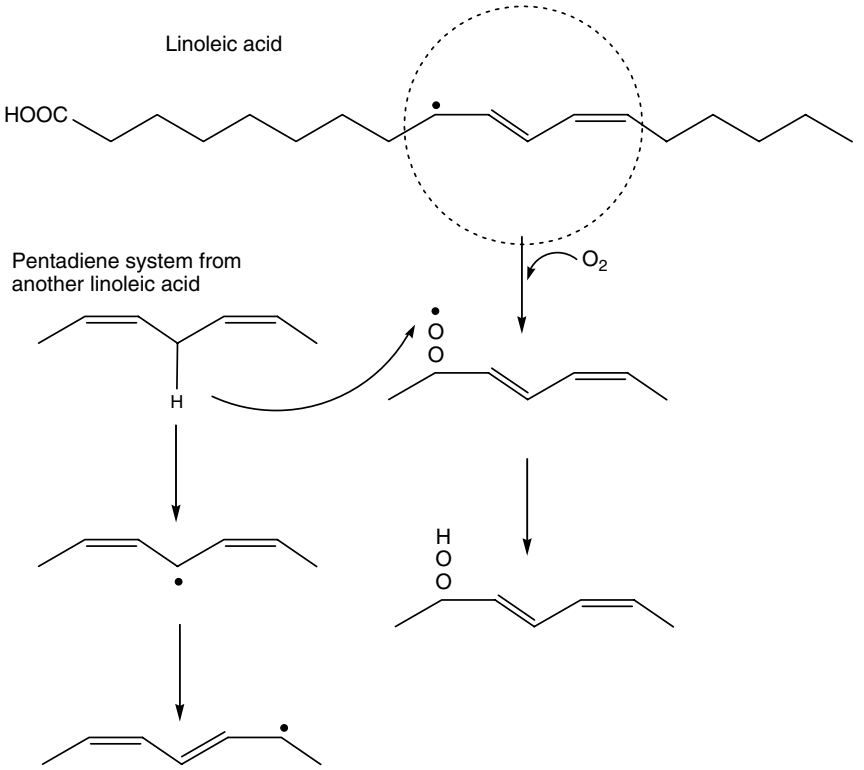


FIGURE 4.25 The propagation step of lipid oxidation for linoleic acid.

Termination: This reaction describes the combination of two radicals to form nonradical species. In the presence of oxygen, the predominant free radical is the peroxyl radical since oxygen will be added onto alkyl radicals at diffusion-limited rates. Thus, under atmospheric conditions, termination reactions may occur between peroxyl and alkoxyl radicals. In low oxygen environments (e.g., frying oils), termination reactions can occur between alkyl radicals to form fatty acid dimers (Figure 4.26). Fatty acid polymers have been used as an indication of frying oil quality [54].

4.7.2 Prooxidants

Lipid oxidation is often referred to as autooxidation. The prefix "auto" means "self-acting," thus the term "autooxidation" has been used to describe the self-perpetuating generation of free radicals from unsaturated fatty acids in the presence of oxygen that occurs during lipid oxidation. In the initiation step, abstraction of hydrogen from unsaturated fatty acids results in the production of a single free radical. The addition of oxygen to the alkyl radical to form a peroxyl radical and subsequent abstraction of hydrogen from another fatty acid or antioxidant to form a lipid hydroperoxide in the propagation step does not result in a net increase in free radicals. Thus, if "autooxidation" was the only reaction in lipid oxidation, the formation of oxidation products would increase linearly from time zero. However, in most foods, the lag phase is followed by a rapid exponential increase in oxidation. This indicates that there are other reactions in lipid oxidation that produce additional free radicals.

Prooxidants, which are found in virtually all food systems, are compounds or factors that cause or accelerate lipid oxidation. Many prooxidants are not true catalysts since they are altered during the reaction (e.g., singlet oxygen is converted to a hydroperoxide and ferrous iron is converted to the ferric state). Prooxidants can accelerate lipid oxidation by direct interactions with unsaturated fatty

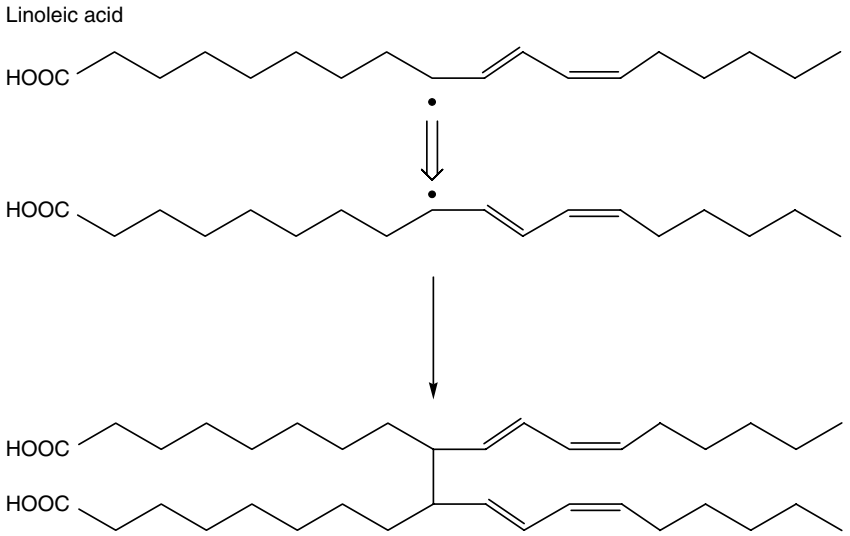


FIGURE 4.26 An example of a termination step of lipid oxidation under conditions of low oxygen concentrations.

acids to form lipid hydroperoxides (e.g., LOXs and singlet oxygen) or by promoting formation of free radicals (e.g., transition metal or UV-light-promoted hydroperoxide decomposition). It should be noted that lipid hydroperoxides do not contribute to off-aromas and thus do not directly cause rancidity. However, hydroperoxides are important substrates for rancidity since their decomposition often results in the scission of the fatty acid to produce low molecular weight volatile compounds responsible for off-aromas. The major prooxidants in foods are discussed below.

4.7.2.1 Prooxidants That Promote Formation of Lipid Hydroperoxides

Singlet oxygen: As mentioned earlier, triplet oxygen ($^3O_2$) is a biradical because its two electrons in the antibonding 2p orbital have the same (parallel or antiparallel) spin direction (Figure 4.27). The Pauli exclusion principle states that two electrons with the same spin direction cannot exist in the same electron orbital. If the electrons in the antibonding 2p orbital have opposite spin directions, oxygen is referred to as singlet oxygen ($^1O_2$). Singlet oxygen can exist in five different configurations with the most common in foods being the $^1\Delta$ state, where the electrons exist in the same orbital (for detailed description see Reference 51). Because singlet oxygen is more electrophilic than triplet oxygen, it can react directly with high electron density double bonds. Since the electrons in singlet oxygen match the spin direction of the electron in double bonds, it can react with an unsaturated fatty acid to directly form lipid hydroperoxides 1500 times faster than triplet oxygen. Singlet oxygen can react with either carbon at the end of a double bond with the double bond then shifting to form a *trans* double bond. This means that oxidation of linoleate by singlet oxygen can produce four different hydroperoxides (Figure 4.27) compared with the typical two hydroperoxides produced in the initiation step of lipid oxidation (Figure 4.22). These different hydroperoxide locations will lead to the formation of several unique fatty acid decomposition products as will be discussed later.

Singlet oxygen is most commonly produced by photosensitization. Chlorophyll, riboflavin, and myoglobin are photosensitizers in foods that can absorb energy from light to form an excited singlet state, which is then converted to an excited triplet state. The excited triplet state can react directly with substrates such as unsaturated fatty acids and abstract a hydrogen to cause initiation of lipid oxidation. This pathway is known as type 1 and will produce the same lipid hydroperoxides seen in

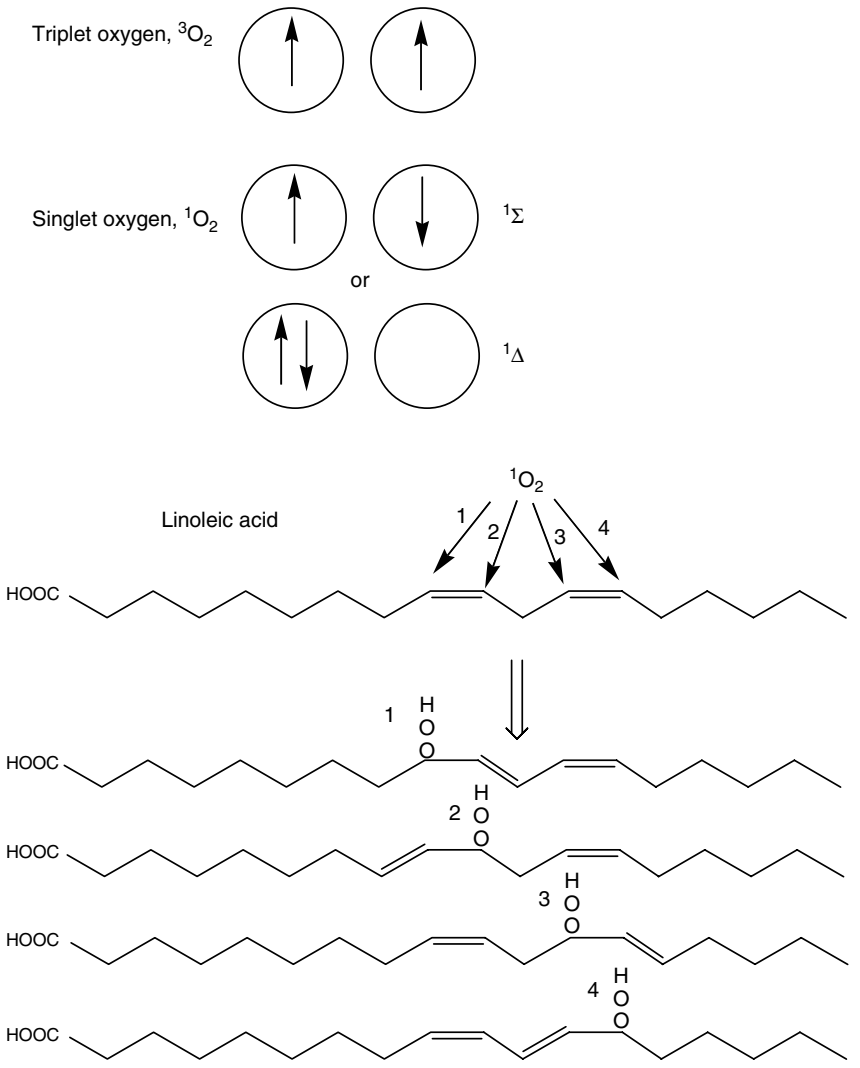


FIGURE 4.27 Singlet oxygen and singlet oxygen-promoted hydroperoxide formation on linoleic acid. (Adapted from Min, D.B. and Boff, J.M. (2002). In *Food Lipids, Chemistry, Nutrition and Biotechnology* (Akoh, C.C. and Min, D.B., eds.), Marcel Dekker, Inc., New York, NY, pp. 335–364.)

the initiation step described in Figure 4.22. The excited triplet state of the photosensitizer can also react with triplet oxygen to form singlet oxygen and singlet state of the photosensitizer in the type 2 pathway. Types 1 and 2 pathways are dependent on oxygen concentrations, with type 2 favored in high oxygen environments. Singlet oxygen can also be formed chemically, enzymatically, and by the decomposition of hydroperoxides. However, production by photosensitization is believed to be the major pathway of singlet oxygen formation in foods.

Lipoxygenase: Numerous plant tissues and animal tissues contain enzymes, known as LOXs that produce lipid hydroperoxides. LOX from plant seeds such as soybeans and peas exist as several isoforms (for review see Reference 55). In soybeans, isoform L-1 primarily reacts with free fatty acids and produces hydroperoxides at carbon 13 in both linoleic and linolenic acid. Isoform L-2 produces hydroperoxides at positions 9 and 13 and is active on both free and esterified linoleic and linolenic acid. Plant LOXs are cytoplasmic enzymes that contain a nonheme iron. The iron in inactive

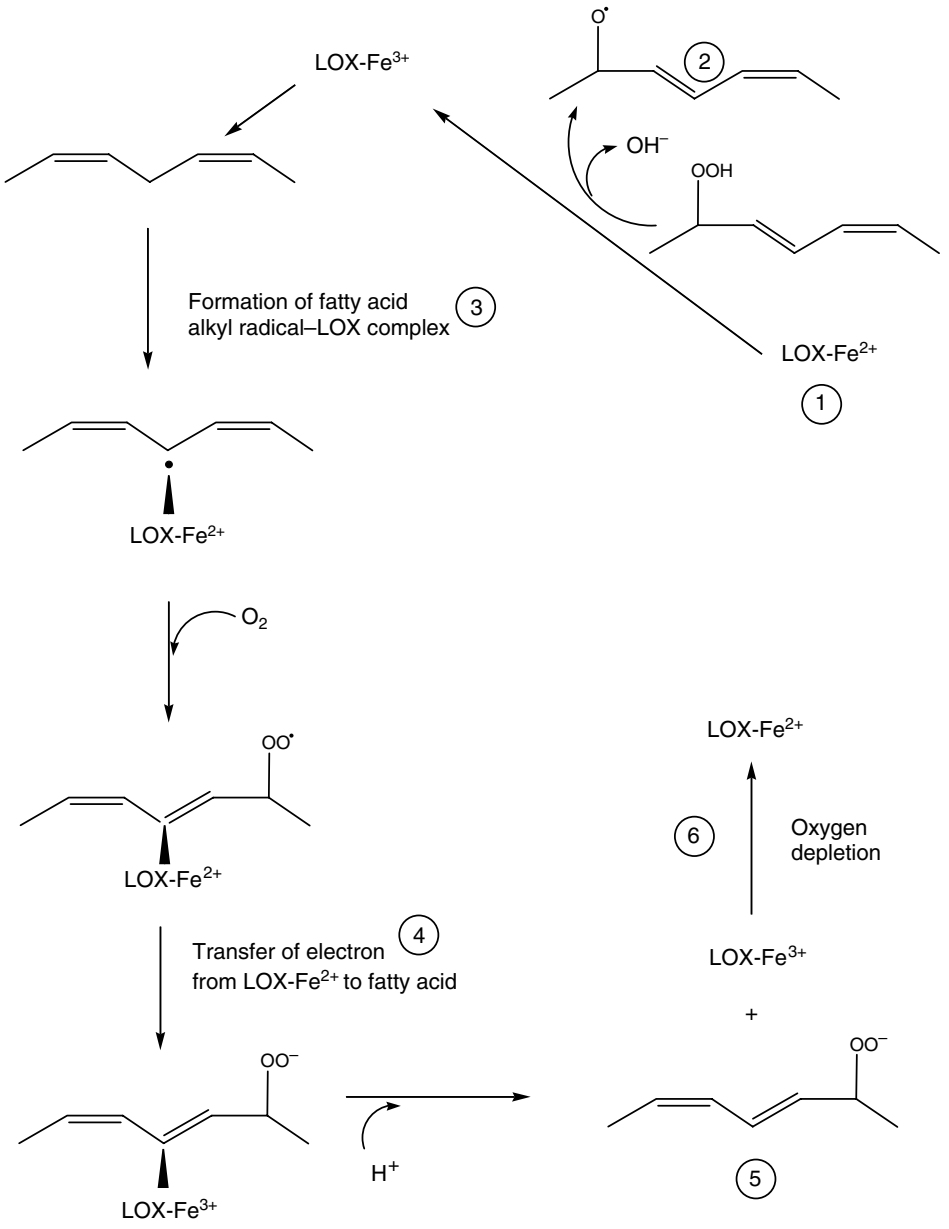


FIGURE 4.28 Mechanism of LOX-promoted hydroperoxide formation on linoleic acid. (Adapted from Zhuang, H., Barth, M.M., and Hildebrand, D. (2002). In *Food Lipids, Chemistry, Nutrition and Biotechnology* (Akoh, C.C. and Min, D.B., eds.), Marcel Dekker, Inc., New York, NY, pp. 413–464.)

LOX is in the ferrous state (Figure 4.28; [1]). Activation occurs by the oxidation of the iron to the ferric state, a process that is usually promoted by a peroxide (2). LOX then catalyzes the abstraction of hydrogen from the methylene-interrupted carbon to form the alkyl radical and the conversion of the LOX iron back to the ferrous state resulting in the formation of a fatty acid alkyl radical–LOX complex (3). An electron from the ferrous iron is then donated to the peroxyl radical to form a peroxyl anion (4). When the peroxyl anion reacts with hydrogen to form the hydroperoxide, the fatty acid is released from the enzyme (5). Once oxygen is depleted from the system, the enzyme abstracts a

hydrogen from a fatty acid and the iron is converted to ferrous (6). Since no oxygen is present, the alkyl radical is released and LOX is returned to its inactive form. LOXs have also been reported in animal tissues especially those highly associated with the circulatory system (e.g., fish gills) [56].

4.7.2.2 Prooxidants That Promote Formation of Free Radicals

Ionizing radiation: Foods are sometimes subjected to ionizing radiation to destroy pathogens and extend shelf-life. However, ionizing radiation can convert molecules to excited states that produce free radicals. Ionizing radiation produces the hydroxyl radical ($^{\bullet}OH$) from water. The hydroxyl radical is the most reactive radical known so it is capable of abstracting hydrogen from lipids as well as molecules such as proteins and DNA. Therefore, it is not surprising that irradiation of foods, especially muscle foods that are high in lipids and prooxidants, can increase oxidative rancidity.

4.7.2.3 Prooxidants That Promote Decomposition of Hydroperoxides

Lipid hydroperoxides are found in essentially all lipid-containing foods. Hydrogen peroxide is also found in food when it is utilized as a processing aid and when it is produced by enzymes such as superoxide dismutase (SOD). Food triacylglycerols typically contain 1–100 nmol lipid hydroperoxide per gram lipid. This is 400–1000 times greater than the estimated lipid hydroperoxide concentrations found *in vivo* (e.g., plasma lipids) suggesting that oxidation occurs during the extraction and refining of fats and oils [57]. Lipid hydroperoxides can be decomposed by high temperatures during thermal processing or by a variety of prooxidants. Upon decomposition they produce additional radicals, a factor that could be responsible for the exponential increase in oxidation that is seen after the lag phase or induction period observed in many foods. The decomposition of lipid hydroperoxides also leads to the formation of alkoxyl radicals that can enter into β-scission reactions. The β-scission reaction is the main pathway responsible for decomposing fatty acids into low molecular weight compounds that are volatile enough to be perceived as oxidative rancidity (discussed below).

Transition metals: Transition metals are found in all foods since they are common constituents of biological materials, water, ingredients, and packaging materials. Transition metals are one of the major food prooxidants that decrease the oxidative stability of foods and biological tissues through their ability to decompose hydroperoxides into free radicals [58,59]. These reactive metals decompose hydrogen and lipid peroxides through the following redox cycling pathway:

$$Mn^{n+} + LOOH \quad \text{or} \quad HOOH \rightarrow Mn^{n+1} + LO^{\bullet} \quad \text{or} \quad HO^{\bullet} + OH^{-} \tag{4.7}$$

$$Mn^{n+1} + LOOH \rightarrow Mn^{n+} + LOO^{\bullet} + H^{+} \tag{4.8}$$

Mn^{n+} and Mn^{n+1} are transition metals in their reduced and oxidized states; LOOH and HOOH are lipid and hydrogen peroxides; and $LO^{\bullet}$, $HO^{\bullet}$, and $LOO^{\bullet}$ are alkoxyl, hydroxyl, and peroxyl radicals, respectively. Hydroxyl radical is produced from hydrogen peroxide while alkoxyl radicals are produced from lipid hydroperoxide. When iron and hydroperoxide are involved in this pathway, it is known as the Fenton reaction. The concentration, chemical state, and type of the metal will influence the rate of hydroperoxide decomposition. Copper and iron are the most common transition metals in foods capable of participating in these reactions with iron generally being found at greater concentrations than copper. Copper is more reactive with the cuprous state (Cu^{1+}) decomposing hydrogen peroxide over 50-fold faster than ferrous ions (Fe^{2+}). Redox state is also important with Fe^{2+} decomposing hydrogen peroxide over 10^5 times faster than Fe^{3+}. In addition, Fe^{2+} is more water soluble than Fe^{3+}, meaning that it is more available to promote hydroperoxide decomposition

in water-based foods. Peroxide type is also important with Fe^{2+} decomposing lipid hydroperoxides about 10 times faster than hydrogen peroxide [58,59].

Since the reduced state of transition metals is more efficient at decomposing hydroperoxides, reducing compounds capable of promoting the redox cycling of transition metals can promote lipid oxidation. Examples of prooxidative reductants include superoxide anion ($^{\bullet}O_2^-$) and ascorbic acid. Superoxide anion is produced by the addition of an electron to triplet oxygen. The added electron in superoxide anion can then be transferred to a transition metal to cause its reduction. Superoxide anion is produced by enzymes, the release of oxygen from oxymyoglobin to produce metmyoglobin, or by cells such as phagocytes. The redox cycling of iron by superoxide anion to promote lipid oxidation is shown in the following pathways. This pathway is known as the Haber–Weiss reaction.

$$Fe^{3+} + {}^{\bullet}O_2^- \rightarrow Fe^{+2} + O_2 \tag{4.9}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{+3} + {}^{\bullet}OH + OH^- \tag{4.10}$$

$$\text{Net} \quad {}^{\bullet}O_2^- + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^- \tag{4.11}$$

Ascorbic acid can also participate in Haber–Weiss-like reactions, however, unlike superoxide anions, ascorbic acid can also act as an antioxidant. At high ascorbate concentration its antioxidant activity dominates its ability to accelerate metal-promoted oxidation resulting in a net antioxidant effect.

Transition metals associated with proteins can also promote hydroperoxide decomposition. The heme proteins are the best studied of this group with the iron in myoglobin, hemoglobin, peroxidases, and catalase being able to promote both hydrogen and lipid hydroperoxide decomposition. In some cases, heme proteins have been suggested to cause homolytic scission of lipid hydroperoxides, meaning that the breakdown of the hydroperoxide will produce two free radicals (hydroxyl and alkoxyl). Thermal denaturation of these proteins can increase their prooxidant activity presumably by increased exposure of the heme iron that is able to more effectively interact with hydroperoxides. Denaturation of myoglobin is one of the factors that accelerate lipid oxidation in cooked meats, a problem known as warmed-over flavor.

Light and elevated temperatures: UV and visible light can promote the decomposition of hydroperoxides to produce free radicals. Thus, packaging to decrease light exposure can attenuate lipid oxidation rates. Elevated temperatures will also decompose lipid hydroperoxides. In fact, lipid hydroperoxide accumulation is often not seen in rancid frying oils since the hydroperoxides breakdown rapidly after formation.

4.7.3 Formation of Lipid Oxidation Decomposition Products

Once lipid hydroperoxides are decomposed into alkoxyl radicals, a number of different reaction schemes can occur. The products of these reaction schemes will depend on the fatty acid type as well as the location of the hydroperoxide on the fatty acid. In addition, decomposition products can be unsaturated and have intact pentadiene structures meaning that the oxidation products can be further oxidized. This results in literally hundreds of different fatty acid decomposition products. Since the type of fatty acid decomposition products will depend on the fatty acid composition of the food, lipid oxidation can have different effects on sensory properties. For example, oxidation of vegetable oils that have predominately ω-6 fatty acids will produce "grassy" and "beany" odors while oxidation of the long-chain ω-3 fatty acids in marine oils will produce "fishy" aromas.

One of the reasons that lipid hydroperoxide decomposition leads to the cleavage of the aliphatic chain of fatty acids is that hydroperoxide decomposition produces the alkoxyl radical ($LO^{\bullet}$). The alkoxyl radical is more energetic than either the alkyl ($L^{\bullet}$) or peroxyl ($LOO^{\bullet}$) radicals. Thus, when

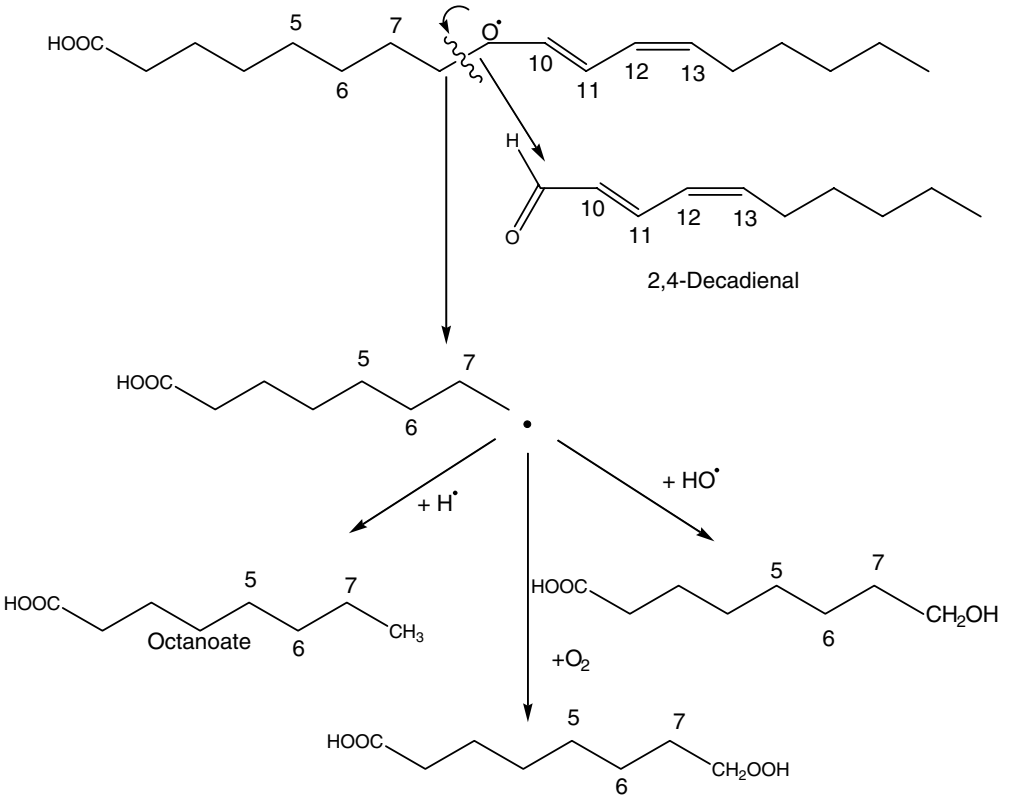


FIGURE 4.29 Possible reaction pathways for a lipid free radical produced by β-scission reactions. (Adapted from Frankel, E.N. (1998). *Lipid Oxidation*, Oily Press, Scotland.)

the alkoxyl radical is produced, it has enough energy to abstract an electron from the covalent bonds adjacent to the alkoxyl radical causing a cleavage of the aliphatic chain of fatty acid. This last reaction, known as the β-scission reaction, is important to food quality since it causes fatty acids to decompose into low molecular weight compounds that are perceived as rancidity.

4.7.3.1 β-Scission Reaction

Decomposition of lipid hydroperoxides to an alkoxyl radicals ($LO^{\bullet}$) is generally accompanied by the β-scission reaction. This reaction breaks the aliphatic chain of the fatty acid to produce aldehydes plus a radical on the aliphatic chain (e.g., an alkyl radical). The alkyl radical can then react with a hydrogen radical to form a hydrocarbon, a hydroxyl radical to form an alcohol or oxygen to form a hydroperoxide. Examples of these reactions are shown in Figure 4.29 and more details of these reactions can be found elsewhere [52]. Since hydroperoxide can form at numerous locations on unsaturated fatty acids, a large number of different products are produced from β-scission reactions. Unesterified linoleic acid will be used to demonstrate the types of products that are produced by β-scission reactions. One should remember that the decomposition product on the carboxylic acid end of the fatty acid would usually be esterified to the glycerol of a triacylglycerol or phospholipid. Thus, this decomposition product would not be volatile and thus, would not contribute to rancidity unless it undergoes further decomposition reactions to form low molecular weight compounds.

Figure 4.30 shows the formation of linoleic acid decomposition products when the hydroperoxide is located at carbon 9 and β-scission occurs on the methyl end side of the molecule. In step 1, the hydroperoxide decomposes into the alkoxyl radical. Step 2 shows the β-scission reaction cleaving the

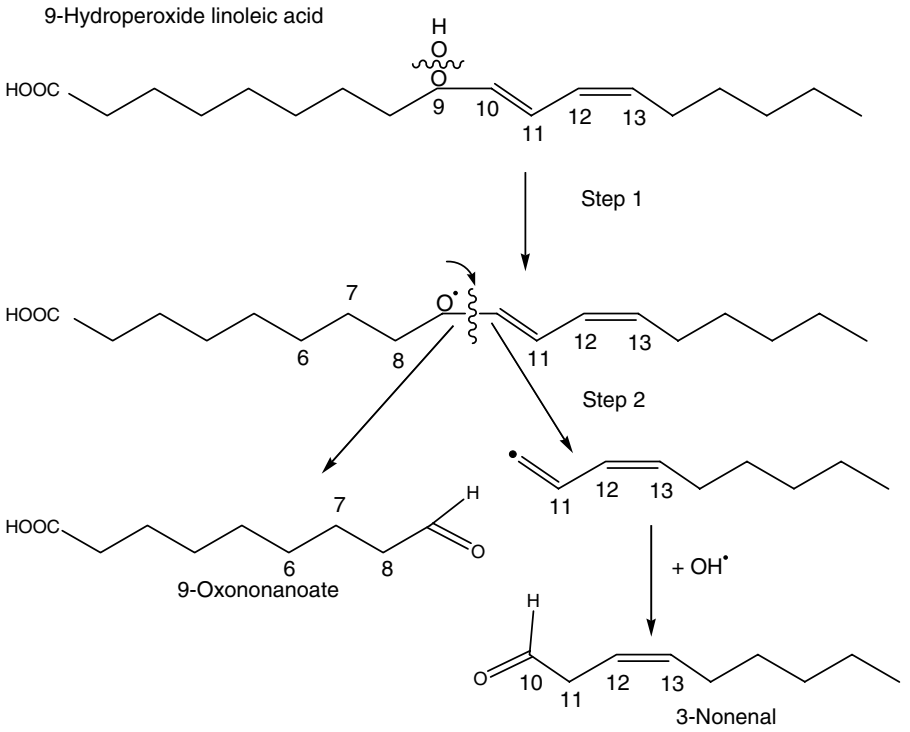


FIGURE 4.30 β-Scission decomposition products produced from 9-linoleic acid hydroperoxide when fatty acid cleavage occurs on the methyl end side of the hydroperoxide. (Adapted from Frankel, E.N. (1998). *Lipid Oxidation*, Oily Press, Scotland.)

adjacent carbon–carbon bonds to form two products. This cleavage (step 2) produces 9-oxononanoate and a nine carbon vinyl radical (an olefinic radical). Vinyl radicals often interact with hydroxyl radicals to form aldehydes, thus producing 3-nonenal. Similar pathways will occur if the hydroperoxide is on carbon 13. Cleavage on the carboxylic acid end will produce 12-oxo-9-dodecenoate and hexanal. Cleavage on the methyl end of the fatty acid will produce 13-oxo-9,11-tridecadienoate and pentane. The 9-linoleic acid hydroperoxide can also undergo β-scission on the carboxylic acid end of the fatty acids, after formation of the alkoxyl radical, as shown in Figure 4.29, to form octanoate and 2,4-decadienal.

When singlet oxygen attacks linoleic acid, it will form hydroperoxides at all of the carbons associated with double bonds (Figure 4.27). This means that it will form hydroperoxides at carbons 9 and 13 as in free radical initiated oxidation plus hydroperoxides at carbons 10 and 12. Typical products from the β-scission reaction from an alkoxyl radical at carbon 10 will produce 9-oxononanoate and 3-nonenal from cleavage on the carboxylic acid end and 10-oxo-8-decenoate and 2-octene from cleavage at the methyl end of the fatty acid. Typical products from the β-scission reaction from an alkoxyl radical at carbon 12 will produce 9-undecenoate and 2-heptenal from cleavage on the carboxylic acid end and 12-oxo-9-dodecenoate and hexanal from cleavage at the methyl end of the fatty acid.

As one can see from the above discussion on the β-scission products and other free radical reactions of linoleic acid, numerous products can be formed. For a detailed discussion on β-scission decomposition products see Reference 52. Pathways similar to this will occur with other unsaturated fatty acids producing additional unique compounds. The decomposition products often contain double bonds and in some cases intact pentadiene systems. These double bond systems can undergo hydrogen abstraction or singlet oxygen attack that will result in the formation of additional

decomposition products. While the above discussion shows the theoretical decomposition products of linoleic acid, in reality, not all of these products have been detected. This is likely owing to the ability of these compounds to undergo additional decomposition reactions.

4.7.3.2 Additional Reactions of Fatty Acid Decomposition Products

In addition to the fatty acid hydroperoxide products described above, fatty acid radicals can undergo a series of other reactions to form compounds such as olefins, alcohols, carboxylic acids, ketones, epoxides, and cyclic products (for review see Reference 52). Alkyl radicals will react with hydrogen and hydroxyl radicals to produce olefins and alcohols. As mentioned earlier, alkoxyl radicals are high energy radicals. Thus, they can abstract hydrogen from other molecules such as unsaturated fatty acids or antioxidants to produce fatty acid alcohols. Alkoxyl radicals can also lose an electron and be converted to a ketone or bond to an adjacent carbon to form an epoxide. Peroxyl radicals can react with double bonds within the same fatty acid to produce cyclic products such as bicyclic endoperoxides.

Aldehydes produced from the oxidative decomposition of fatty acids are important because of their impact on off-flavor development. However, these aldehydes can react with nucleophilic food components. In particular, they interact with sulfhydryls and amines in proteins that may alter the functionality of the protein. One example is the ability of unsaturated aldehydes to react with histidine in myoglobin via a Michael addition-type reaction [60]. This reaction is thought to contribute to the conversion of myoglobin to metmyoglobin to produce meat discoloration.

4.7.3.3 Cholesterol Oxidation

Cholesterol contains a double bond between carbons 5 and 6. As with fatty acids, this double bond is susceptible to free radical attack and can undergo decomposition reactions to produce alcohols, ketones, and epoxides [61]. The most notable of the cholesterol oxidation pathway begins with the formation of a hydroperoxide at carbon 7. This hydroperoxide can decompose into an alkoxyl radical that can in turn undergo rearrangements to 5,6 epoxides, 7-hydroxylcholesterol, and 7-ketocholesterol. These cholesterol oxidation products are potentially cytotoxic and have been linked to the development of atherosclerosis. Cholesterol oxidation products have primarily been found in animal food products that have undergone thermal processing such as cooked meats, tallow, lard, and butter, as well as dried dairy and egg products.

4.7.4 Antioxidants

Oxidative stress occurs in all organisms in an oxygenated environment. Thus biological systems have developed a variety of antioxidant defenses to protect against oxidation. There is no a uniform definition of an antioxidant because there are numerous chemical mechanisms by which oxidation can be inhibited. The biological tissue from which foods is obtained, generally contain several endogenous antioxidant systems. Unfortunately, food processing operations can remove antioxidants or cause oxidative stress that can overcome the endogenous antioxidants systems in the food. Therefore, it is common to incorporate additional antioxidant protection into processed foods. Antioxidant mechanisms of compounds that are used to increase the oxidative stability of foods include control of free radicals, prooxidants, and oxidation intermediates.

4.7.4.1 Control of Free Radicals

Many antioxidants slow lipid oxidation by scavenging free radicals, thus inhibiting initiation, propagation, and β-scission reactions. Free radical scavengers (FRSs) or chain-breaking antioxidants

can interact with peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals by the following reactions:

$$LOO^{\bullet} \quad \text{or} \quad LO^{\bullet} + FRS \rightarrow LOOH \quad \text{or} \quad LOH + FRS^{\bullet} \tag{4.12}$$

Free radical scavengers inhibit lipid oxidation by reacting faster with free radicals than unsaturated fatty acids. FRSs are thought to interact mainly with peroxyl radicals because their lower energy state means they have a longer lifetime (because they are less reactive) and thus, have a greater likelihood of reacting with the low energy hydrogen FRS. This is contrary to high energy free radicals (e.g., $^{\bullet}OH$) that are so reactive they interact with the molecules closest to their site of production. Since FRSs are generally found at low concentrations they would be less likely to react with the high energy free radicals [62].

Antioxidant efficiency is dependent on the ability of the FRS to donate hydrogen to a free radical. As bond energy of a hydrogen in a FRS decreases, the transfer of the hydrogen to the free radical is more energetically favorable and thus more rapid. The ability of a FRS to donate its hydrogen to a free radical can be predicted with the help of standard one-electron reduction potentials [63]. Any compound that has a reduction potential lower than the reduction potential of a free radical (or oxidized species) is capable of donating its hydrogen to that free radical unless the reaction is kinetically unfeasible. For example, FRS including α-tocopherol ($E^{\circ\prime} = 500$ mV), catechol ($E^{\circ\prime} = 530$ mV), and ascorbate ($E^{\circ\prime} = 282$ mV), all have reduction potentials below peroxyl radicals ($E^{\circ\prime} = 1000$ mV) and are therefore capable of donating their hydrogen to the peroxyl radical to form a hydroperoxide.

The efficiency of the FRS is also dependent on the energy of the resulting FRS radical ($FRS^{\bullet}$). If the $FRS^{\bullet}$ is a low energy radical then the likelihood of the $FRS^{\bullet}$ catalyzing the oxidation of unsaturated fatty acids decreases. Effective FRS forms low energy radicals owing to resonance delocalization (as shown in Figure 4.31). Effective FRS also produce radicals that do not react rapidly with oxygen to form hydroperoxides. If a radical scavengers form a hydroperoxide, it could undergo decomposition reactions that produce additional radicals that could cause oxidation of unsaturated fatty acids. $FRS^{\bullet}$ may participate in termination reactions with other $FRS^{\bullet}$ or lipid radicals to form nonradical species. This means that each FRS is capable of inactivating at least two free radicals, the first being inactivated when the FRS interacts with peroxyl or alkoxyl radicals and the second when the $FRS^{\bullet}$ enters a termination reactions with another $FRS^{\bullet}$ or lipid radical (Figure 4.32).

Phenolic compounds possess many of the properties of an efficient FRS. Phenolic compounds donate a hydrogen from their hydroxyl groups and the subsequent phenolic radical can have low energy as the radical is delocalized throughout the phenolic ring structure. The effectiveness of phenolic FRS is often increased by substitution groups on the phenolic ring that increase the ability of the FRS to donate hydrogen to lipid radicals and/or increase the stability of the $FRS^{\bullet}$ [64]. In foods, the efficiency of phenolic FRS is also dependent on their volatility, pH sensitivity, and polarity. Below are examples of the most common FRS in foods.

Tocopherols: Tocopherols are a group of compounds that have a hydroxylated ring system (chromanol ring) with a phytol chain (Figure 4.33). Differences in tocopherol homologs are because of differences in methylation on the chromanol ring with α being trimethylated (positions 5, 7, and 8), β (positions 5 and 8) and γ (positions 7 and 8) being dimethylated, and δ being monomethylated (position 8). Tocotrienols differ from tocopherols in that they have three double bonds in their phytol chain at positions $3'$, $7'$, and $11'$. Tocopherols have three asymmetric carbons and thus each homolog can have eight possible stereoisomers. Natural tocopherols are found in the all *rac* or *RRR* configuration. Synthetic tocopherols have stereoisomer with combinations of *R* and *S* configurations. The stereoisomer configuration of α-tocopherol is important because only the *RRR* and 2*R*-stereoisomers (*RSR*, *RRS*, and *SRR*) have significant vitamin E activity and can be used for establishment of the Dietary Reference Intake of vitamin E in the United States [65]. α-Tocopherol is commonly sold as a methyl ester when used as a nutritional supplement. The methyl ester is hydrolyzed in the gastrointestinal

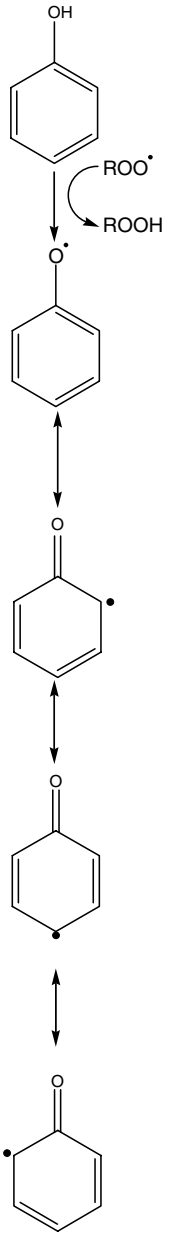


FIGURE 4.31 Resonance delocalization of phenol radical. (Adapted from Shahidi, F. and Wanasundara, J.P.K. (1992). *Crit. Rev. Food Sci. Nutr.* 32: 67–103.)

tract to regenerate α-tocopherol. The methyl ester form of tocopherols blocks the hydroxyl group and decreases the molecule's susceptibility to oxidative degradation until digested. It should be noted that the blocking of the hydroxyl group by the methyl ester removes the antioxidant activity of the tocopherol. Therefore, methyl esters of tocopherols would not be effective antioxidants in foods.

Reaction between tocopherols and lipid peroxyl radicals leads to the formation of a lipid hydroperoxide and several resonance structures of tocopheroxyl radicals. These tocopheroxyl radicals can interact with other lipid radicals or with each other to form a variety of termination products. The types and amounts of these products are dependent on oxidation rates, radical species, physical location

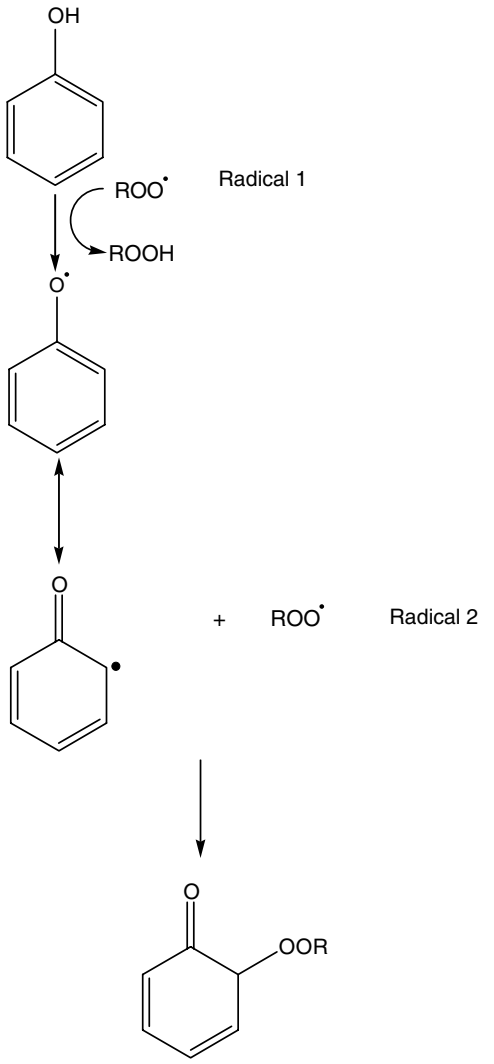


FIGURE 4.32 A termination reaction between an antioxidant radical and a lipid peroxyl radical ($ROO^{\bullet}$).

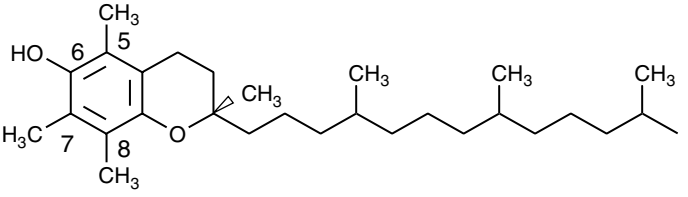


FIGURE 4.33 The structure of α-tocopherol.

(e.g., bulk vs. membrane lipids), and tocopherol concentration (see Reference 62 for more details). Tocopherols are generally insoluble in water. However, they do vary in polarity, with α-tocopherol (trimethylated) being the most nonpolar and δ-tocopherol (monomethylated) being the most polar. These differences in polarity alter the surface activity of the tocopherols, a factor that may impact their antioxidant activity (see Section 4.7.4.5).

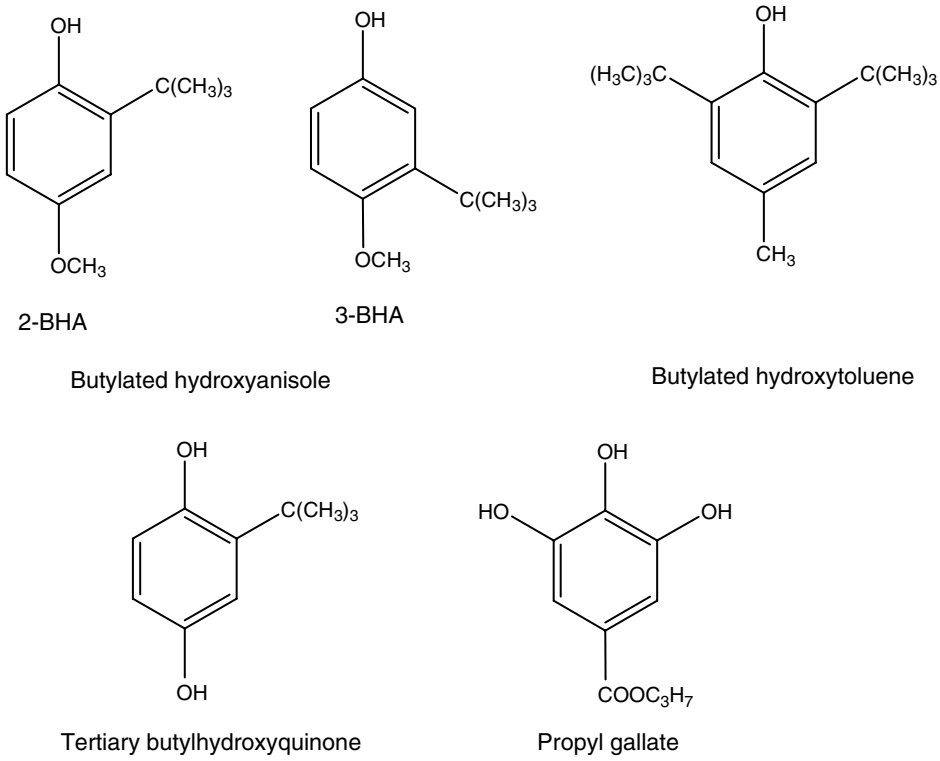


FIGURE 4.34 Structures of synthetic antioxidants used in foods.

Synthetic phenolics: Phenol is not a good antioxidant but addition of substitution groups onto the phenolic ring can enhance antioxidant activity. Thus, the majority of synthetic antioxidants are substituted monophenolic compounds. The most common synthetic FRSs used in foods include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and propyl gallate (Figure 4.34). These synthetic FRSs vary in polarity in the order of BHT (most nonpolar) > BHA > TBHQ > propyl gallate (see Section 4.7.4.5 for an explanation of the importance of antioxidant polarity). As with other FRSs, interactions between the synthetic antioxidants and lipid radicals result in the formation of a low energy resonance-stabilized phenolic radical. The low energy of the synthetic antioxidant radicals means that they do no rapidly catalyze the oxidation of unsaturated fatty acids. In addition, synthetic antioxidant radicals do not react readily with oxygen to form unstable antioxidant hydroperoxides, which decompose into high energy free radicals that can promote oxidation. Instead, they tend to react in radical–radical termination reactions, as shown in Figure 4.32. Synthetic phenolics are effective in numerous food systems; however, their use in the food industry has recently declined owing to safety concerns and consumer demand for all natural products.

Plant phenolics: Plants contain a diverse group of phenolic compounds including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids. These phenolics are widely distributed in fruits, spices, tea, coffee, seeds, and grains. All of the phenolic classes have the structural requirements of FRSs although their activity varies greatly. Factors influencing the FRS activity of plant phenolics include position and degree of hydroxylation, polarity, solubility, reducing potential, stability of the phenolic to food processing operations, and stability of the phenolic radical. Rosemary extracts are the most commercially important source of natural phenolics used as a food additive to inhibit lipid oxidation by FRS. Carnosic acid, carnosol, and rosmarinic acid are the major

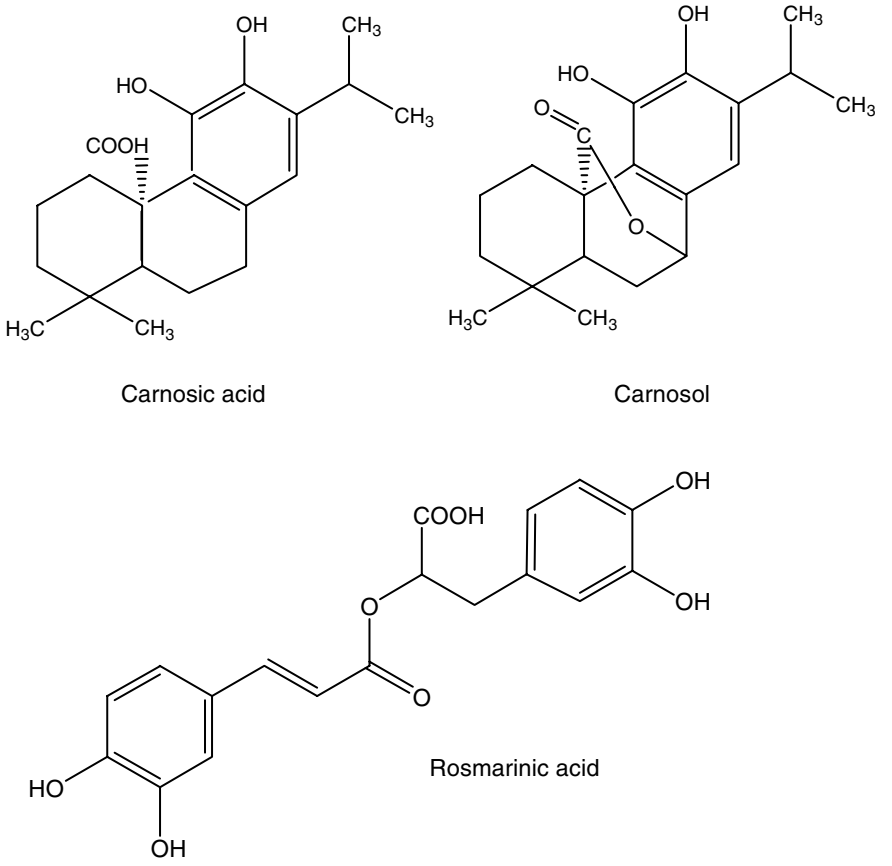


FIGURE 4.35 Structures of phenolic antioxidants found in rosemary extracts.

FRSs in rosemary extracts (Figure 4.35). Rosemary extracts can inhibit lipid oxidation in a wide variety of food products including meats, bulk oils, and lipid emulsions [66–68]. Utilization of phenolic antioxidants from crude herb extracts, such as rosemary, is often limited by the presence of flavor compounds such as monoterpenes. Phenolics found naturally in plant foods and oils are important to the endogenous oxidative stability of foods. Phenolic levels in plants can vary as a function of plant maturity, variety, tissue type, growing conditions, and postharvest age and storage conditions [69–71].

Ascorbic acid and thiols: Free radicals are often generated in the water phase of foods by processes such as the Fenton reaction that produces hydroxyl radicals from hydrogen peroxide. Free radicals may also be surface active, meaning that they could migrate or partition at interface between the lipid phase and water phase in lipid dispersions. To protect against free radicals derived in the aqueous phase, biological systems contain water-soluble compounds capable of free radical scavenging. Ascorbic acid and thiols scavenge free radicals resulting in the formation of low energy radicals (for review see Reference 72). Thiols such as cysteine and glutathione may contribute to the oxidative stability of plant and muscle foods but they are rarely added to foods as antioxidants. One exception to this is the thiols found in proteins that can inhibit lipid oxidation in food products [72]. Ascorbate and its isomer erythorbic acid can both scavenge free radicals. Both have similar activity but erythorbic acid is more cost effective. Ascorbic acid is also available as a conjugate with palmitic acid. The conjugate is lipid soluble and surface active, and this makes it an effective antioxidant in bulk oils

and emulsions. In the gastrointestinal tract ascorbyl palmitate is hydrolyzed to ascorbic and palmitic acid, thus there are no restrictions on its usage levels.

4.7.4.2 Control of Prooxidants

The rate by which lipids oxidize in foods is very dependent on prooxidant concentrations and activity (e.g., transition metals, singlet oxygen, and enzymes). Control of prooxidants is therefore a very effective strategy to increase the oxidative stability of foods. Both endogenous and exogenous antioxidants will impact the activity of transition metals and singlet oxygen.

Control of prooxidant metals: Iron and copper are examples of important prooxidant transition metals that accelerate lipid oxidation by promoting hydroperoxide decomposition. The prooxidative activity of metals is altered by chelators or sequestering agents. Chelators inhibit the activity of prooxidant metals by one or more of the following mechanisms: prevention of metal redox cycling; occupation of all metal coordination sites; formation of insoluble metal complexes; and/or steric hindrance of interactions between metals and lipids or oxidation intermediates (e.g., hydroperoxides) [73]. Some metal chelators can increase oxidative reactions by increasing metal solubility and/or altering the redox potential. The tendency of a chelator to accelerate or inhibit prooxidant activity depends on metal-to-chelator ratio. For instance, EDTA (ethylenediamine tetraacetic acid) is ineffective or prooxidative when EDTA:iron ratios are ≤ 1 and antioxidative when EDTA:iron is >1 [74]. The prooxidative behavior of chelators is thought to be because of their ability to increase transition metal solubility.

The main metal chelators found in foods contain multiple carboxylic acid (e.g., EDTA and citric acid) or phosphate groups (e.g., polyphosphates and phytate). Chelators must be ionized to be active; therefore their activity decreases at pH values below the pK_a of the ionizable groups. The most common chelators used as food additives are citric acid, EDTA, and polyphosphates. The effectiveness of phosphates increases with increasing number of phosphate groups; thus, tripolyphosphate and hexametaphosphate are more effective than phosphoric acid [75]. Prooxidant metals can also be controlled by metal binding proteins, such as transferrin, phosvitin, lactoferrin, ferritin, and casein (reviewed in Reference 73).

Control of singlet oxygen: As mentioned earlier, singlet oxygen is an excited state of oxygen that can promote the formation of lipid hydroperoxides. Carotenoids are a diverse group (>600 different compounds) of yellow to red colored polyenes. The activity of singlet oxygen can be controlled by carotenoids by both chemical and physical quenching mechanisms [76,77]. Carotenoids chemically quench singlet oxygen when singlet oxygen attacks the double bonds of the carotenoid. This reaction leads to the formation of oxygenated carotenoid breakdown products such as aldehydes, ketones, and endoperoxides. These reactions cause carotenoid decomposition, leading to loss of color. The more effective mechanism of singlet oxygen inactivation by carotenoids is physical quenching. Carotenoids physically quench singlet oxygen by a transfer of energy from singlet oxygen to the carotenoid to produce an excited state carotenoid and ground state triplet oxygen. Energy is dissipated from the excited carotenoid by vibrational and rotational interactions with the surrounding solvent to return the carotenoid to the ground state. The nine or more conjugated double bonds in a carotenoid are necessary for physical quenching. Carotenoids that have oxygenated β-ionone ring structures at the ends are often more effective at physically quenching singlet oxygen. Carotenoids can also physically absorb the energy of photoactivated sensitizers such as riboflavin preventing the photosensitizer from promoting the formation of singlet oxygen.

Control of LOXs: Lipoxygenases are active lipid oxidation catalysts found in plants and some animal tissues. LOX activity can be controlled by heat inactivation and plant breeding programs that decrease the concentration of these enzymes in edible tissues.

4.7.4.3 Control of Oxidation Intermediates

Compounds are found in foods that indirectly influence lipid oxidation rates by interacting with prooxidant metals or oxygen to form reactive species. Examples of such compounds include superoxide anion and hydroperoxides.

Superoxide anion: Superoxide participates in oxidative reactions by reducing transition metals to a more active state or by promoting the release of iron bound to protein. In addition, at pH values below its pK_a (i.e., 4.8), superoxide will form the perhydroxyl radical ($HOO^{\bullet}$) that can directly catalyze lipid oxidation [78]. Owing to the prooxidant nature of superoxide anion in oxidative reactions, biological systems contain SOD. SOD catalyzes the conversion of superoxide anion to hydrogen peroxide by the following reaction:

$$2^{\bullet}O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \tag{4.13}$$

Peroxides: Peroxides are important intermediates of oxidative reactions since they decompose via transition metals, irradiation, and elevated temperatures to form free radicals. Hydrogen peroxide exists in foods owing to direct addition (e.g., aseptic processing operations) and formation in biological tissues by mechanisms including the dismutation of superoxide by SOD and the activity of peroxisomes and leukocytes. The inactivation of hydrogen peroxide is catalyzed by catalase, a heme-containing enzyme, by the following reaction [78]:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{4.14}$$

Glutathione peroxidase is a selenium-containing enzyme that can decompose both lipid hydroperoxides and hydrogen peroxide using reduced glutathione (GSH) as a cosubstrate [78]:

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \tag{4.15}$$

or

$$LOOH + 2GSH \rightarrow LOH + H_2O + GSSG \tag{4.16}$$

where GSSG is oxidized glutathione and LOH is a fatty acid alcohol.

4.7.4.4 Antioxidant Interactions

Food systems usually contain endogenous multicomponent antioxidant systems. In addition, exogenous antioxidants can be added to processed foods. The presence of multiple antioxidants will enhance the oxidative stability of the product owing to interactions between antioxidants. Synergism is often used to describe antioxidant interactions. For antioxidant interactions to be synergistic, the effect of the antioxidant combination must be greater than the sum of the two individual antioxidants. However, in most cases the effectiveness of antioxidant combinations often is equal to or less than their additive effect. While antioxidant combinations can be used to effectively increase the shelf-life of foods, caution should be used in claiming synergistic activity.

Enhanced antioxidant activity can be observed in the presence of two or more different FRSs. In the presence of multiple FRSs, it is possible that one FRS (the primary FRS) will react more rapidly with lipid free radicals than the others owing to lower bond dissociation energies or owing to the fact that its physical location is closer to the site where free radicals are being generated. In the presence of multiple FRSs, the primary FRS, which is rapidly oxidized, can sometimes be regenerated by a secondary FRS with the free radical being transferred from the primary to the secondary FRS. This process is seen with α-tocopherol and ascorbic acid. In this system, α-tocopherol is the primary FRS owing to its presence in the lipid phase. Ascorbic acid then regenerates the tocopheroxyl

radical or possibly the tocopherylquinone back to α-tocopherol, resulting in the formation of the dehydroascorbate [63]. The net result is that the primary FRS (α-tocopherol) is maintained in an active state where it can continue to scavenge free radicals in the lipid phase of the food.

Chelator and FRS combinations can result in improved inhibition of lipid oxidation [6]. These enhanced interactions occur by a "sparing" effect provided by the chelator. That is, the chelator decreases the amount of free radicals formed in the food by inhibiting metal-catalyzed oxidation; this slows down inactivation of the FRS through reactions such as termination or autooxidation.

Since multicomponent antioxidant systems can inhibit oxidation by many different mechanisms (e.g., FRS, metal chelation, and singlet oxygen quenching), the use of multiple antioxidants can greatly enhance the oxidative stability of foods. Thus, when designing antioxidant systems, the antioxidants used should have different mechanisms of action and/or physical properties. Determining which antioxidants would be most effective depends on factors such as type of oxidation catalysts, physical state of the food, and factors that influence the activity of the antioxidants themselves (e.g., pH, temperature, and ability to interact with other compounds/antioxidants in the foods).

4.7.4.5 Physical Location of Antioxidants

Antioxidants can show a wide range of effectiveness depending on the physical nature of the lipid [52,79]. For example, hydrophilic antioxidants are often less effective in O/W emulsions than lipophilic antioxidants, whereas lipophilic antioxidants are less effective in bulk oils than hydrophilic antioxidants. This observation has been coined the "polar paradox." Differences in the effectiveness of the antioxidants in bulk oils and W/O emulsions are due to their physical location in the two systems. Polar antioxidants are more effective in bulk oils presumably because they can accumulate at the air–oil interface or in reverse micelles within the oil, the locations where lipid oxidation reactions would be greatest owing to high concentrations of oxygen and prooxidants. In contrast, predominantly, nonpolar antioxidants are more effective in O/W emulsions because they are retained in the oil droplets and/or may accumulate at the oil–water interface, the location where interactions between hydroperoxides at the droplet surface and prooxidants in the aqueous phase occur. Conversely, in O/W emulsions, polar antioxidants would tend to partition into the continuous aqueous phase where they would be less able to protect the lipid.

4.7.5 Other Factors Influencing Lipid Oxidation Rates

Oxygen concentration: Reduction of oxygen concentration is a common method used to inhibit lipid oxidation. However, the addition of oxygen to the alkyl radical is a diffusion-limited (fast) reaction; therefore, to effectively inhibit lipid oxidation, the majority of oxygen must be removed from the system. Since oxygen solubility is higher in oil than in water, removal of oxygen to stop lipid oxidation can be difficult unless vacuum conditions are used or oxygen is completely replaced by an inert gas (e.g., nitrogen).

Temperature: Increasing temperature generally increases lipid oxidation rates. However, increasing temperature also decreases oxygen solubility so in some cases high temperatures can slow oxidation. This can happen in heated bulk oil. However, if food is fried in heated oil, aeration of the oil occurs leading to acceleration of oxidation. Elevated temperatures can also cause antioxidants to degrade, volatilize, and, in the cases of antioxidant enzymes, become inactivated through denaturation.

Surface area: Increasing the surface area of lipids can increase lipid oxidation rates since this can lead to increased exposure to oxygen and prooxidants.

Water activity: As water is removed from a food system, lipid oxidation rates generally decrease. This is likely because of a decrease in the mobility of reactants such as transition metals and oxygen. In some foods, continued removal of water will result in an acceleration of lipid oxidation. This

acceleration of lipid oxidation at low water activity ($a_w \leq 0.3$) is thought to be due to the loss of a protective water solvation layer surrounding lipid hydroperoxides [80].

4.7.6 Measurement of Lipid Oxidation

As one can glean from the above discussion on lipid oxidation pathways, numerous oxidation products can be formed from a single fatty acid. In addition, these decomposition products often contain double bonds and in some cases intact pentadiene systems. These double bond systems can undergo further hydrogen abstraction or singlet oxygen attack that will result in the formation of additional decomposition products. Since food lipids can contain many different unsaturated fatty acids and can be exposed to several different prooxidants, hundreds of decomposition products can be formed. The complexity of these pathways makes analysis of lipid oxidation very challenging. Below is a summary of the most common analytical techniques to monitor the oxidation products in food lipids.

4.7.6.1 Sensory Analysis

The gold standard of lipid oxidation measurements is sensory analysis since this is the only technique that directly monitors the off-aromas and off-flavors generated by oxidative reactions. In addition, sensory analysis can be highly sensitive since humans can detect certain aroma compounds at levels below or close to detection levels for chemical and instrumental techniques. Sensory analysis of oxidized lipids must be done with a panel that is trained in the identification of oxidation products. This training is usually product specific since the oxidation products from different fatty acids can produce different sensory profiles. Owing to the necessity for extensive training, sensory analysis is often time consuming and cost prohibitive and obviously is not suitable for the rapid and extensive analysis required for quality control operations. Thus, many chemical and instrumental techniques have been developed. In the best-case scenario, these chemical and instrumental techniques are most useful when correlated with sensory analysis. Numerous tests exist for measurement of oxidative deterioration of foods. The most common methods and their advantages and disadvantages are discussed below.

4.7.6.2 Primary Lipid Oxidation Products

Primary lipid oxidation products are compounds that are produced by the initiation and propagation steps of lipid oxidation. Since these are the first oxidation products produced, they can appear early in the oxidative deterioration of lipids. However, during the latter stages of oxidation, the concentrations of these compounds decrease as their formation rates become slower than their decomposition rates. A disadvantage of using primary products to measure oxidation is that primary products are not volatile and, thus, do not directly contribute to off-flavors and off-aromas. In addition, under certain conditions (such as high temperatures [frying oils] or high amounts of reactive transition metals), the concentration of primary products may show little net increase since their decomposition rates are relatively high. This would produce misleading results since a very rancid oil could have very low concentrations of primary lipid oxidation products.

Conjugated double bonds: Conjugated double bonds are rapidly formed in polyunsaturated fatty acids upon the abstraction of hydrogen in the initiation step. Conjugated dienes have an absorption maximum at 234 nm with a molar extinction coefficient of 2.5×10^4 M^{-1} cm^{-1} [81]. This extinction coefficient gives an intermediate level of sensitivity compared to other techniques. Conjugated dienes can be useful for simple oil systems, however, it is often ineffective in complex foods where many compounds exist that also absorb at similar wavelengths and thus cause interference. Sometimes, conjugated diene values are used interchangeably with lipid hydroperoxides since many lipid hydroperoxides will contain a conjugated diene system. However, this equivalence should be

discouraged since fatty acid breakdown products can also contain conjugated double bonds and since monounsaturated fatty acids (e.g., oleic) will form hydroperoxides that do not have a conjugated diene system. Conjugated trienes can also be measured in foods at 270 nm. This technique is only useful with lipids that have $\geq$3 double bonds and, thus, is limited to highly unsaturated oils such as those from linseed and fish.

Lipid hydroperoxides: A very common method to measure the oxidative quality of lipids is to measure fatty acid hydroperoxides. Most methods that measure lipid hydroperoxides rely on the ability of the hydroperoxides to oxidize an indicator compound. Peroxide values are expressed as milliequivalents (mEq) of oxygen per kg of oil with 1 mEq equal to 2 mmol of hydroperoxide. The most common titration method uses the hydroperoxide-promoted conversion of iodide to iodine. Iodine is then titrated with sodium thiosulfite to produce iodide that is measured with a starch indicator [82]. This method is relatively insensitive with a detection limit of 0.5 mEq kg^{-1} oil and can require up to 5 g of lipid. Thus, it is only practical for isolated or bulk fats and oils. Lipid hydroperoxide-promoted oxidation of ferrous to ferric ions can also be used with ferric ions being detected with ferric ion-specific chromophores such as thiocyanate or xylenol orange [83]. These methods are much more sensitive than the iodine sodium thiosulfite titration methods. The chromophore formed from the thiocyanate–ferric complex has an extinction coefficient of 4.0×10^4 M^{-1} cm^{-1} allowing analysis to be performed with milligram quantities of lipids [83].

4.7.6.3 Secondary Lipid Oxidation Products

Secondary lipid oxidation products are compounds that arise from the decomposition of fatty acid hydroperoxides via reactions such as β-scission. As described above, these reactions can generate hundreds of different compounds, both volatile and nonvolatile, from the oxidation of food lipids. Since it is virtually impossible to measure all of these compounds simultaneously, these methods (see below) generally focus on the analysis of a single compound or class of compounds. A drawback of these methods is that the formation of secondary products derives from the decomposition of lipid hydroperoxides. Thus, in certain cases (e.g., presence of antioxidants), the concentrations of secondary products can be low while primary oxidation product concentrations are high. In addition, compounds in foods containing amine and sulfhydryl groups (e.g., proteins) can interact with secondary products that contain functional groups such as aldehydes, thus making them difficult to measure. An advantage of these measurements is that they measure many of the products from fatty acid decomposition, which are directly responsible for the off-flavors and off-aromas in rancid oils, and thus have higher correlation with sensory analysis.

Analysis of volatile secondary products: Volatile lipid oxidation products are typically measured by gas chromatography using direct injection, static or dynamic headspace, or solid-phase microextraction (SPME) [84]. Using these systems, lipid oxidation can be measured using specific products (e.g., hexanal for lipids high in ω-6 fatty acids and propanal for lipids high in ω-3 fatty acids), product classes (e.g., hydrocarbons or aldehydes), or by total volatiles as indicators. Each method can give different profiles of volatiles owing to differences in their ability to extract and collect the volatiles from the sample. The advantage of measuring volatile lipid oxidation products is the strong correlation with sensory analysis. The disadvantage is expense of instrumentation and the difficulty in analyzing large amounts of samples especially in lipids that are oxidizing rapidly (these techniques are often time consuming). In addition, these methods often use heating steps to increase the concentration of volatiles in the headspace above the samples. In some foods, such as meats, these heating steps may increase lipid oxidation rates by cooking the food. In general, lipids should be sampled at the lowest temperature possible. An additional problem is the loss of volatile compounds by processes such as steam distillation in frying oils.

Carbonyls: Carbonyls arising from lipid oxidation can be determined by reacting lipids with 2,4-dinitrophenylhydrazine to form corresponding hydrazones that absorb light at 430–460 nm.

This method is limited by the presence of other carbonyls in foods that can cause interference [82]. High performance liquid chromatography (HPLC) techniques have been developed to separate carbonyls arising from lipid oxidation from interfering compounds. However, these techniques are sophisticated and time consuming, and therefore not routinely used in food lipids.

Carbonyls can also be measured by conjugation with anisidine to form products that absorb at 350 nm [52]. This method is useful because it can measure nonvolatile, high molecular weight carbonyls. This makes it a useful method for frying oils where volatile oxidation products are lost by steam distillation. Anisidine is also used to measure oxidation in products such as fish oils since these oils commonly undergo extensive steam distillation during refining. Anisidine is therefore useful in fish oils because it can give an indication of the quality of the oil prior to steam distillation since nonvolatile, high molecular weight compounds are retained in the oil.

Thiobarbituric acid (TBA): The TBA assay is based on the reaction between TBA and carbonyls to form red, fluorescent adducts under acidic conditions [85]. The assay can be conducted on whole samples, sample extracts, or sample distillates, and adduct formation can be conducted under over a range of protocol temperatures (25–100°C) and times (15 min to 20 h). The compound often attributed to be the primary lipid oxidation product detected by TBA is malondialdehyde (MDA) whose TBA adduct absorbs strongly at 532 nm. MDA is a dialdehyde produced by a two-step oxidative degradation of fatty acids with three or more double bonds. This means that MDA yield during the oxidation of lipids is dependent on fatty acid composition with highly unsaturated fatty acids producing higher amounts of MDA. TBA can also react with aldehydic lipid oxidation products other than MDA, especially unsaturated aldehydes.

The TBA assay suffers from nonspecificity owing to its ability to react with nonlipid carbonyls such as ascorbic acid, sugars, and nonenzymatic browning products. These compounds can form TBA adducts that absorb over the range of 450–540 nm. Often it is more appropriate to refer to TBA reactive substances (TBARS to acknowledge that compounds in addition to MDA can generate pink chromophores). In order to decrease problems with interfering compounds, the TBA–MDA complex can be measured directly by fluorescence or HPLC techniques.

The TBA assay can be a useful method for analysis of lipid oxidation in foods since it is simple and inexpensive. However, the nonspecificity of the method requires an understanding of the test's limitations so that improper comparisons and conclusions are not made. To minimize potential misinterpretation of TBA analysis, it is suggested that analysis of fresh, nonoxidized samples be conducted to account for TBA reactive substances that do not arise from lipid oxidation. However, the TBA method should be avoided in foods where concentrations of interfering compounds are high. In addition, attempts to use TBA to compare oxidative changes in products with different fatty acid compositions are inappropriate since MDA yield varies with fatty acid composition.

4.8 FOOD LIPIDS AND HEALTH

4.8.1 Bioactivity of Fatty Acids

Dietary lipids have often been negatively associated with health. Since obesity is highly correlated with numerous diseases, such as heart disease and diabetes, the negative role of lipids in health is often attributed to their high caloric density of 9 kcal g^{-1}. Specific dietary lipids have also been associated with risk of heart disease owing to their ability to modulate LDL-cholesterol levels in the blood. This includes the saturated fatty acids that increase LDL-cholesterol levels and the unsaturated fatty acids that decrease LDL cholesterol. Since LDL-cholesterol levels are often associated with the development of heart disease, several dietary strategies have been proposed to decrease LDL cholesterol including reduction of dietary saturated fatty acids to $<7\%$ of calories, reduction of dietary cholesterol to <200 mg per day, and incorporation of dietary soluble fibers at 10–25 g per day [86].

4.8.1.1 *Trans* Fatty Acids

Trans fatty acids have recently gained attention in their unique role in heart disease through their ability to both increase LDL-cholesterol and decrease high-density lipoprotein (HDL) cholesterol [87]. This behavior is partially due to the geometric configuration of *trans* fatty acids that is more similar to saturated fatty acids than unsaturated fatty acids. Originally, *trans* fatty acids are included in the unsaturated fatty acid category of the nutritional label in the United States even though their biological activities are very different. As of January 1, 2006, all foods are required to list *trans* fatty acid concentrations on their nutritional labels. Foods with less than 0.5 g of fat/serving do not have to label *trans* fatty acids as long as no claims are made about fat, fatty acids, or cholesterol content.

While a large amount of research has been devoted to the negative aspects of dietary lipids on health, evidence is growing that some dietary lipids can reduce the risk to several diseases. These bioactive lipids include ω-3 fatty acids, phytosterols, carotenoids, and CLA.

4.8.1.2 ω-3 Fatty Acids

As agricultural practices have advanced, the profile of dietary lipids in Western societies has changed dramatically. Our ancestors are thought to have consumed diets with approximately equal amounts of ω-6 and ω-3 fatty acids. The development of modern agriculture increased the availability of refined fats, especially vegetable oils, changing our dietary ω-6 to ω-3 ratio to over 7:1. This is an extremely rapid change on an evolutionary timescale that is problematic since humans interconvert ω-6 and ω-3 fatty acids at slow rates. Levels of ω-3 fatty acids in the diet are important because these bioactive lipids play a vital role in membrane fluidity, cellular signaling, gene expression, and eicosanoid metabolism. Therefore, consumption of dietary ω-3 fatty acids is essential for promotion and maintenance of good health, especially for pregnant and lactating women, and individuals with coronary heart disease, diabetes, immune response disorders, and compromised mental health. There is strong evidence that the level of ω-3 fatty acids currently consumed by the general population is inadequate [9]. Numerous food companies are attempting to increase the levels of these bioactive lipids in their products by direct incorporation of ω-3 fatty acids into foods or by feeding ω-3 fatty acids to livestock. These approaches are commonly impeded by the oxidative deterioration of the ω-3 fatty acids during the processing and storage of fortified food products. A list of marine foods high in ω-3 fatty acids is shown in Table 4.5. Seed oils rich in ω-3 fatty acids, specifically linolenic acid, include soybean, canola, and flaxseed (linseed) (Table 4.2).

TABLE 4.5
The ω-3 Fatty Acid Content of Selected Fish

Fish	g ω-3 Fatty Acids/100 g Fish
Tuna (white albacore)	0.9
Tuna (light)	0.2
Atlantic salmon (farmed)	1.3–2.1
Chinook salmon (wild)	1.4
Herring	2.0
Mackerel	0.4–1.8
Cod	0.2
Flounder	0.5
Catfish	0.1

Source: Exler, J. (1987). *Composition of Foods: Finfish and Shellfish Products*. USDA Handbook 8-15, Washington, DC.

4.8.1.3 Conjugated Linoleic Acid

The two double bonds of linoleic acid are normally in a methylene-interrupted system where two single bonds separate the double bonds. However, the double bond system is sometimes altered resulting in isomerization of the double bonds to a conjugated configuration. This isomerization can occur during processes such as hydrogenation and is common during the biohydrogenation process promoted by bacteria in ruminants. These isomers, known as CLA, have gained widespread interest because of their ability to inhibit cancer [89], lower blood cholesterol [90], inhibit the onset of diabetes, and influence weight gain [91]. The different isomers have different biological effects with 9-*cis*, 11-*trans* linoleic acid showing anticarcinogenic activity and 10-*trans*, 12-*cis* linoleic acid having the ability to influence body fat accumulation. The 9-*cis*, 11-*trans* isomer of CLA is the predominant isomer found in dairy and beef products. Molecular mechanisms of CLA bioactivity have been attributed to their ability to modulate eicosanoid formation and gene expression. Very few human clinical studies have been conducted to support the purported health benefits of CLA in humans.

4.8.1.4 Phytosterols

The major phytosterols in foods are sitosterol, campesterol, and stigmasterol. Dietary phytosterols are practically nonabsorbed in the gastrointestinal tract. Their bioactivity lies in the fact that they can inhibit the absorption of both dietary and biliary (produced by intestinal cells) cholesterol [92]. The intake of 1.5–2 g per day of phytosterols can reduce LDL-cholesterol by 8–15%. Since phytosterols primarily inhibit cholesterol absorption, they are most effective when consumed with a cholesterol-containing meal. Phytosterols have very high melting points and thus exist as lipid crystals at the temperatures common to many foods. To minimize crystallization, the phytosterols are commonly esterified to unsaturated fatty acids to increase their lipid solubility.

4.8.1.5 Carotenoids

Carotenoids are a diverse group (>600 different compounds) of yellow to red colored polyenes that are lipid soluble. Vitamin A is an essential nutrient obtained from carotenoids such as β-carotene. The bioactivity of other carotenoids has been a research area of great interest. This interest was initially focused on the antioxidant activity of carotenoids. However, when clinical trials were conducted to evaluate dietary β-carotene in subjects at risk to free radical stress (smokers), β-carotene was found to increase lung cancer rates [93]. It is unknown whether a similar effect would be observed with nonsmokers. Other carotenoids have been found to have health benefits. Lutein and zeaxanthin can enhance visual acuity and health [94]. Epidemiological studies have shown that the consumption of tomatoes is correlated with decreased risk for prostate cancer [95]. The health benefits of tomatoes have been attributed to the carotenoid, lycopene. Interestingly, cooked tomatoes have greater health benefits presumably owing to the thermally induced conversion of *trans*-lycopene to *cis*-lycopene isomers. The greater bioactivity of *cis*-lycopene isomers is thought to be because of their greater bioavailability.

4.8.2 Low Calorie Lipids

One of the other health concerns of dietary triacylglycerols is their high caloric density. Many attempts have been made to produce low fat foods that have the same sensory attributes as their full fat counterparts by using fat mimetics. Fat mimetics are nonlipid compounds such as proteins or carbohydrates that can produce fat-like properties at lower caloric values (e.g., 4 kcal g^{-1} protein vs. 9 kcal g^{-1} lipid). A similar approach has been attempted to produce lipid components with no calories or with lower caloric contents (fat substitutes). The first commercial noncaloric lipid was sucrose fatty acid esters (Proctor and Gamble's Olestra). This compound is noncaloric because the presence of $\geq$6 fatty acids esterified to sucrose sterically prevents lipase from hydrolyzing the ester

bond to release free fatty acids that can be absorbed into the blood. The nondigestiability of sucrose fatty acid esters means that they pass through the gastrointestinal tract and are excreted in the feces. This property can cause gastrointestinal problems such as diarrhea. Structured lipids with lower caloric density have also been used in the food industry (e.g., Nabisco's Salatrim). These products are based on the principle that only fatty acids at *sn*-1 and *sn*-3 of triacylglycerol are released as free fatty acids on hydrolysis by pancreatic lipase. If *sn*-1 and *sn*-3 have long-chain saturated fatty acids ($\geq$16 carbons), their release can lead to interactions with divalent cations to form insoluble soaps that are not readily bioavailable. Structured low calorie fats also use short-chain fatty acids ($\leq$6 carbons) at the *sn*-2 position. After hydrolysis by pancreatic lipase, the *sn*-2 monoacylglycerol is absorbed into the intestinal endothelial cells. The short-chain fatty acids at *sn*-2 eventually are metabolized in the liver where they yield fewer calories than long-chain fatty acids. The combination of both long-chain saturated fatty acids at *sn*-1 and *sn*-3 and short-chain fatty acids at *sn*-2 produce a triacylglycerol with 5–7 cal g^{-1}.

4.9 SUMMARY

Food lipids play an important role in food quality by contributing to attributes such as texture, flavor, nutrition, and caloric density. As knowledge of the nutritional importance of lipids continue to evolve, manufacturers will need to modify the physical and chemical properties of food lipids in order to produce healthy foods with high consumer acceptability. This means that foods will likely be produced to contain less of the nutritionally detrimental lipids (e.g., lower in total fat, saturated fat, and *trans* fatty acids). However, to accomplish these goals, food chemists will need to have a strong understanding of how lipids impact texture and flavor. Food will also be produced to contain nutritionally beneficial lipids such as ω-3 fatty acids and phytosterols. A thorough understanding of the physical properties and chemical stability of lipids will be required to produce functional foods with bioactive lipids since these compounds can be chemically unstable (ω-3 fatty acids) or difficult to physically incorporate into foods.

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5 Amino Acids, Peptides, and Proteins

Srinivasan Damodaran

CONTENTS

5.1 INTRODUCTION

Proteins play a central role in biological systems. Although the information for evolution and biological organization of cells is contained in DNA, enzymes exclusively perform the chemical and biochemical processes that sustain the life of a cell/organism. Thousands of enzymes have been discovered. Each one of them catalyzes a highly specific biological reaction in cells. In addition to functioning as enzymes, proteins (such as collagen, keratin, elastin, etc.) also function as structural components of cells and complex organisms. The functional diversity of proteins essentially arises from their chemical make up.

Proteins are highly complex polymers, made up of 21 different amino acids. The constituents are linked via substituted amide bonds. Unlike the glycosidic and phosphodiester bonds in polysaccharides and nucleic acids, respectively, the amide linkage in proteins is a partial double bond, which further underscores the structural complexity of protein polymers. The myriad biological functions performed by proteins might not be possible but for the complexity in its composition, which gives rise to a multitude of three dimensional structural forms with different biological functions. To signify their biological importance, these macromolecules were named proteins, derived from the Greek word *proteois*, which means of the first kind.

At the elemental level, proteins contain on wt/wt basis 50–55% carbon, 6–7% hydrogen, 20–23% oxygen, 12–19% nitrogen, and 0.2–3.0% sulfur. Protein synthesis occurs in ribosomes. After synthesis, cytoplasmic enzymes modify some amino acid constituents. This changes the elemental composition of some proteins. Proteins that are not enzymatically modified in cells are called *homoproteins*, and those that are modified or complexed with nonprotein components are called *conjugated proteins* or *heteroproteins*. The nonprotein components are often referred to as *prosthetic groups*. Examples of conjugated proteins include *nucleoproteins* (ribosomes), *glycoproteins* (ovalbumin and κ-casein), *phosphoproteins* (α- and β-caseins, kinases, and phosphorylases), *lipoproteins* (proteins of egg yolk and several plasma proteins), and *metalloproteins* (hemoglobin, myoglobin and several enzymes). Glyco- and phosphoproteins contain covalently linked carbohydrate and phosphate groups, respectively, whereas the other conjugated proteins are noncovalent complexes containing nucleic acids, lipids, or metal ions. These complexes can be dissociated under appropriate conditions.

Proteins also can be classified according to their gross structural organization. Thus, *globular proteins* are those that exist in spherical or ellipsoidal shapes, resulting from folding of the polypeptide chain(s) on itself. On the other hand, *fibrous proteins* are rod-shaped molecules containing twisted linear polypeptide chains (e.g., tropomyosin, collagen, keratin, and elastin). Fibrous proteins also can be formed as a result of linear aggregation of small globular proteins, for example, actin and fibrin. A majority of enzymes are globular proteins, and fibrous proteins invariably function as *structural proteins*.

The various biological functions of proteins can be categorized as *enzyme catalysts*, *structural proteins*, *contractile proteins* (myosin, actin, and tubulin), *hormones* (insulin and growth hormone), *transfer proteins* (serum albumin, transferrin, and hemoglobin), *antibodies* (immunoglobulins), *storage proteins* (egg albumen and seed proteins), and protective proteins (toxins and allergens). Storage proteins are found mainly in eggs and plant seeds. These proteins act as sources of nitrogen and amino acids for germinating seeds and embryos. The protective proteins are a part of the defense mechanism for the survival of certain microorganisms and animals.

All proteins are essentially made up of the same primary 20 amino acids; however, some proteins do not contain all 20 amino acids. The differences in structure and function of these thousands of proteins arise from the sequence in which the amino acids are linked together via amide bonds. Literally, billions of proteins with unique properties can be synthesized by changing the amino acid sequence, the type and ratio of amino acids, and the chain length of polypeptides.

All biologically produced proteins can be used as *food proteins.* However, for practical purposes, *food proteins* may be defined as those that are easily digestible, nontoxic, nutritionally adequate,

functionally useable in food products, available in abundance, and sustainable agriculturally. Traditionally, milk, meats (including fish and poultry), eggs, cereals, legumes, and oilseeds have been the major sources of food proteins. These are mainly storage proteins in animal and plant tissues, which act as the nitrogen source for the growing embryo. However, because of the burgeoning world population, nontraditional sources of proteins for human nutrition need to be developed to meet the future demand. The suitability of such new protein sources for use in foods, however, depends on their cost and their ability to fulfill the normal role of protein ingredients in processed and domestically prepared foods.

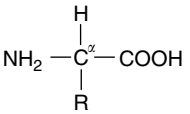
The functional properties of proteins in foods are related to their structural and other physicochemical characteristics. A fundamental understanding of the physical, chemical, nutritional, and functional properties of proteins and the changes these properties undergo during processing is essential if the performance of proteins in foods is to be improved, and if new or less costly sources of proteins are to compete with traditional food proteins.

5.2 PHYSICOCHEMICAL PROPERTIES OF AMINO ACIDS

5.2.1 General Properties

5.2.1.1 Structure and Classification

α-Amino acids are the basic structural units of proteins. These amino acids consist of a α-carbon atom covalently attached to a hydrogen atom, an amino group, a carboxyl group, and a side chain R group.



(5.1)

Natural proteins contain up to 21 different primary amino acids linked together via amide bonds. The 21st new amino acid, which has been recognized as a natural amino acid, is selenocysteine [12]. These amino acids differ only in the chemical nature of the side chain R group (Figure 5.1). The physicochemical properties, such as net charge, solubility, chemical reactivity, and hydrogen bonding potential, of the amino acids are dependent on the chemical nature of the R group.

The amino acids listed in Figure 5.1 have genetic codes, including selenocysteine. That is, each one of these amino acids has a specific *t*-RNA that translates the genetic information on *m*-RNA into an amino acid sequence during protein synthesis. Apart from the 21 primary amino acids listed in Figure 5.1, several proteins also contain other types of amino acids, which are derivatives of the primary amino acids. These *derived amino acids* are either cross-linked amino acids or simple derivatives of single amino acids. Proteins that contain derived amino acids are called *conjugated* proteins. Cystine, which is found in most proteins, is a good example of a cross-linked amino acid. Other cross-linked amino acids, such as desmosine, isodesmosine, and di- and trityrosine, are found in structural proteins such as elastin and resilin. Several simple derivatives of amino acids are found in several proteins. For example, 4-hydroxyproline and 5-hydroxylysine are found in collagen. These are the result of posttranslational modification during maturation of collagen fiber. Phosphoserine and phosphothreonine are found in several proteins, including caseins. *N*-Methyllysine is found in myosin, and γ-carboxy-glutamate is found in several blood clotting

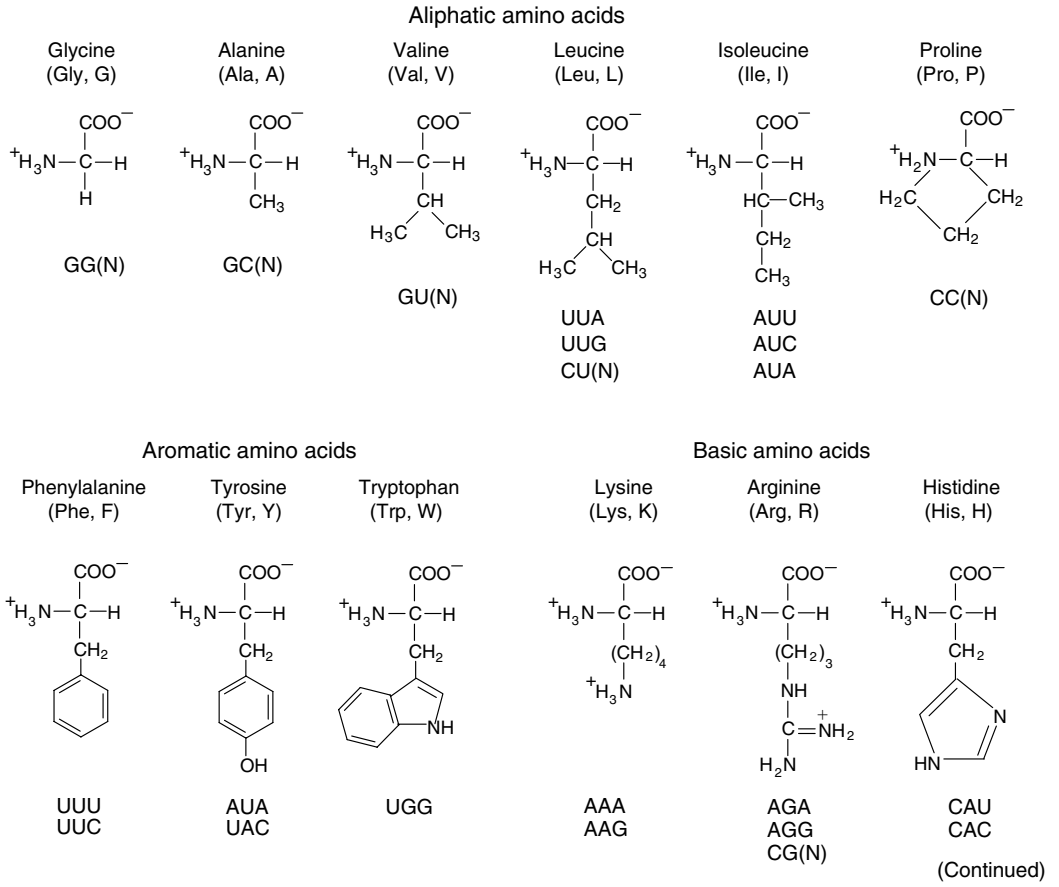
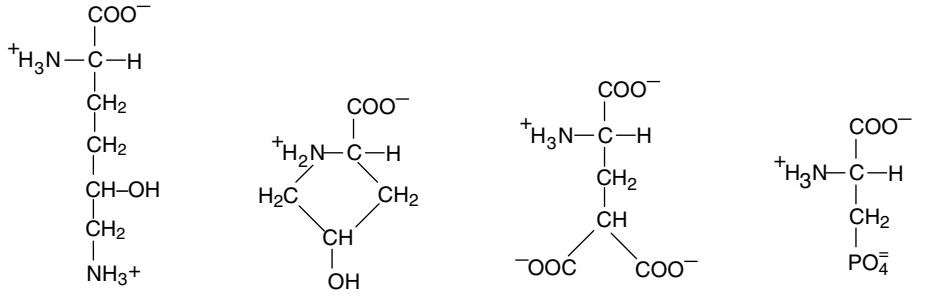


FIGURE 5.1 Primary α-amino acids that occur in proteins. The three letter and one letter codes of amino acids are shown in parenthesis. The mRNA codons for the amino acids are also shown for each amino acid.

factors and calcium binding proteins:



(5.2)

5.2.1.2 Stereochemistry of Amino Acids

With the exception of Gly, the α-carbon atom of all amino acids is asymmetric, meaning that four different groups are attached to it. Because of this asymmetric center, amino acids exhibit optical

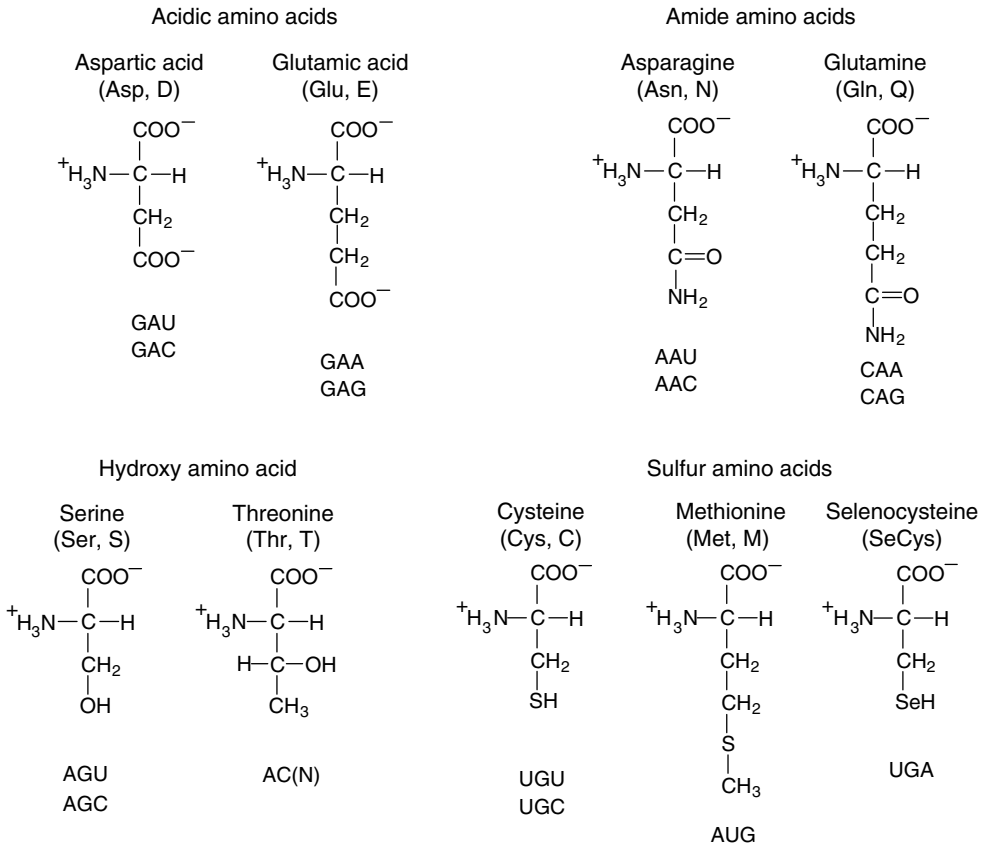
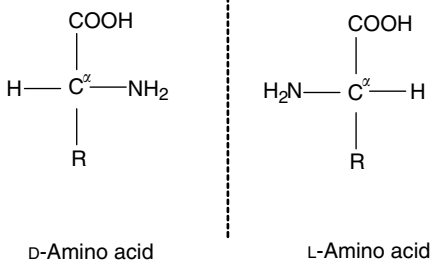


FIGURE 5.1 Continued.

activity, that is, they rotate the plane of linearly polarized light. In addition to the asymmetric α-carbon atom, the β-carbon atoms of Ile and Thr are also asymmetric, and thus both Ile and Thr can exist in four enantiomeric forms. Among the derived amino acids, hydroxyproline and hydroxylysine also contain two asymmetric carbon centers. All proteins found in nature contain only L-amino acids. Conventionally, the L- and D-enantiomers are represented as

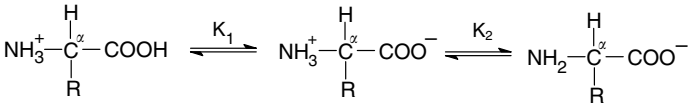


(5.3)

This nomenclature is based on D- and L-glyceraldehyde configurations and not on the actual direction of rotation of linearly polarized light. That is, the L-configuration does not refer to levorotation as in the case of L-glyceraldehyde. In fact most of the L-amino acids are dextrorotatory, not levorotatory.

5.2.1.3 Acid–Base Properties of Amino Acids

Since amino acids contain a carboxyl group (acidic) and an amino group (basic), they behave both as acids and bases; that is, they are *ampholytes*. For example, Gly, the simplest of all amino acids, can exist in three different ionized states, depending on the pH of the solution.



(5.4)

At around neutral pH, both the α-amino and α-carboxyl groups are ionized, and the molecule is a *dipolar* or a *zwitter ion*. The pH at which the dipolar ion is electrically neutral is called the *isoelectric point* (pI). When the zwitter ion is titrated with an acid, the COO^- group becomes protonated. The pH at which the concentrations of COO^- and COOH are equal is known as pK_{a1} (i.e., negative logarithm of the acid dissociation constant K_{a1}). Similarly, when the zwitter ion is titrated with a base, the NH_3^+ group becomes deprotonated. As before, the pH at which $[NH_3^+] = [NH_2]$ is known as pK_{a2}. A typical electrometric titration curve for a dipolar amino acid is shown in Figure 5.2. In addition to the α-amino and α-carboxyl groups, the side chains of Lys, Arg, His, Asp, Glu, Cys, and Tyr also contain ionizable groups. The pK_a values of all the ionizable groups in amino acids are given in Table 5.1. The isoelectric points of amino acids can be estimated from their pK_{a1}, pK_{a2}, and pK_{a3} values, using the following expressions:

For amino acids with no charged side chain, $\quad pI = (pK_{a1} + pK_{a2})/2$

For acidic amino acids, $\quad pI = (pK_{a1} + pK_{a3})/2$, and

For basic amino acids, $\quad pI = (pK_{a2} + pK_{a3})/2$

The subscripts 1, 2, and 3 refer to α-carboxyl, α-amino, and side chain ionizable groups, respectively.

In proteins, the α-COOH of one amino acid is covalently coupled to the α-NH_2 of the next amino acid through an amide bond, thus the only ionizable groups in proteins are the N-terminus amino

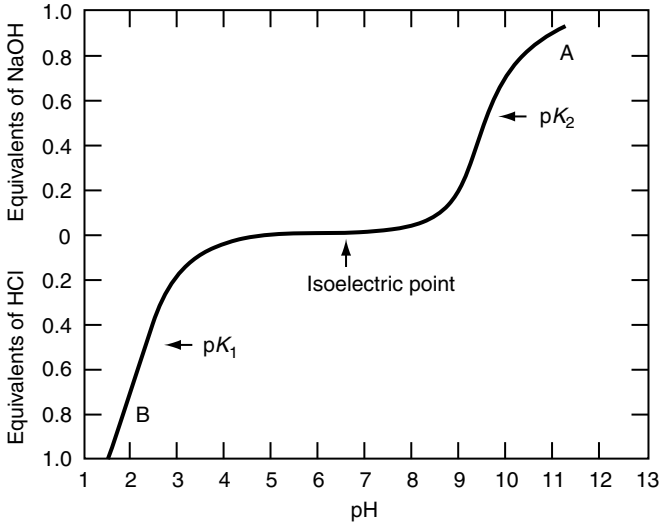


FIGURE 5.2 Titration curve of a typical amino acid.

TABLE 5.1
Properties of Ionizable Groups in Free Amino Acids at 25°C

Amino Acid	pK_{a1} •(—COOH)	pK_{a2} •($—NH_3^+$)	pK_{a3} (Side Chain)	pI
Alanine	2.34	9.69	—	6.00
Arginine	2.17	9.04	12.48	10.76
Asparagine	2.02	8.80	—	5.41
Aspartic acid	1.88	9.60	3.65	2.77
Cysteine	1.96	10.28	8.18	5.07
Glutamine	2.17	9.13	—	5.65
Glutamic acid	2.19	9.67	4.25	3.22
Glycine	2.34	9.60	—	5.98
Histidine	1.82	9.17	6.00	7.59
Isoleucine	2.36	9.68	—	6.02
Leucine	2.30	9.60	—	5.98
Lysine	2.18	8.95	10.53	9.74
Methionine	2.28	9.21	—	5.74
Phenylalanine	1.83	9.13	—	5.48
Proline	1.94	10.60	—	6.30
Serine	2.20	9.15	—	5.68
Threonine	2.21	9.15	—	5.68
Tryptophan	2.38	9.39	—	5.89
Tyrosine	2.20	9.11	10.07	5.66
Valine	2.32	9.62	—	5.96

group, the C-terminus carboxyl group, and ionizable groups on side chains. The pK_a of the ionizable groups in proteins are different from those of free amino acids (Table 5.2). The significant shift in the pK_a values in proteins as compared to free amino acids is related to altered electronic and dielectric environments of these groups in proteins. (This property is important in enzymes.)

The degree of ionization of a group at any given solution pH can be determined by using the Henderson–Hasselbach equation:

$$\text{pH} = pK_a + \log \frac{[\text{conjugated base}]}{[\text{conjugated acid}]} \tag{5.5}$$

The net charge of a protein at a given pH can be estimated by determining the degree of ionization of individual ionizable groups using this equation, and then adding up the total number of negative and positive charges.

Amino acids may be classified into several categories based on the nature of interaction of the side chains with water. Amino acids with aliphatic (Ala, Ile, Leu, Met, Pro, and Val) and aromatic side chains (Phe, Trp, and Tyr) are hydrophobic, and hence they exhibit limited solubility in water (Table 5.3). Polar (hydrophilic) amino acids are quite soluble in water and they are either charged (Arg, Asp, Glu, His, and Lys) or uncharged (Ser, Thr, Asn, Gln, and Cys). The side chains of Arg and Lys contain guanidyl and amino groups, respectively, and thus are positively charged (basic) at neutral pH. The imidazole group of His is basic in nature. However, at neutral pH its net charge is only slightly positive. The side chains of Asp and Glu acids contain a carboxyl group. These amino acids carry a net negative charge at neutral pH. Both the basic and acidic amino acids are strongly hydrophilic. The net charge of a protein at physiological conditions is dependent on the relative numbers of basic and acidic amino acids residues in the protein.

TABLE 5.2
Average pK_a Values of Ionizable Groups in Proteins

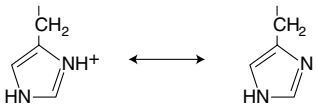
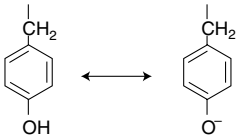
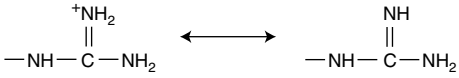
Ionizable Group	pK_a	Acid Form ⟷ Base Form
Terminal COOH	3.75	$-COOH \longleftrightarrow -COO^-$
Terminal NH_2	7.8	$-NH_3^+ \longleftrightarrow -NH_2$
Side chain COOH (Glu, Asp)	4.6	$-COOH \longleftrightarrow -COO^-$
Side chain NH_2	10.2	$-NH_3^+ \longleftrightarrow -NH_2$
Imidazole	7.0	
Sulfhydryl	8.8	$-SH \longleftrightarrow -S^-$
Phenolic	9.6	
Guanidyl	>12	

TABLE 5.3
Properties of Amino Acids at 25°C

Amino Acid	Molecular Weight	Residue Volume Δ^3	Residue Area Δ^2	Solubility (g/L)	Hydrophobicity (kcal/mol)[a] (ΔG_{tr}^0)
Ala	89.1	89	115	167.2	0.4
Arg	174.2	173	225	855.6	−1.4
Asn	132.1	111	150	28.5	−0.8
Asp	133.1	114	160	5.0	−1.1
Cys	121.1	109	135	—	2.1
Gln	146.1	144	180	7.2(37°C)	−0.3
Glu	147.1	138	190	8.5	−0.9
Gly	75.1	60	75	249.9	0
His	155.2	153	195	—	0.2
Ile	131.2	167	175	34.5	2.5
Leu	131.2	167	170	21.7	2.3
Lys	146.2	169	200	739.0	−1.4
Met	149.2	163	185	56.2	1.7
Phe	165.2	190	210	27.6	2.4
Pro	115.1	113	145	620.0	1.0
Ser	105.1	89	115	422.0	−0.1
Thr	119.1	116	140	13.2	0.4
Trp	204.2	228	255	13.6	3.1
Tyr	181.2	194	230	0.4	1.3
Val	117.1	140	155	58.1	1.7

[a] The ΔG values are relative to glycine based on the side-chain distribution coefficients (K_{eq}) between 1-octanol and water [41].

The polarities of uncharged neutral amino acids fall between those of hydrophobic and charged amino acids. The polar nature of Ser and Thr is attributed to the hydroxyl group that is able to hydrogen bond with water. Since Tyr also contains an ionizable phenolic group, which ionizes at alkaline pH, it is also considered to be a polar amino acid. However, based on its solubility characteristics at neutral pH, it should be regarded as a hydrophobic amino acid. The amide group of Asn and Gln is able to interact with water through hydrogen bonding. Upon acid or alkaline hydrolysis, the amide group of Asn and Gln is converted to a carboxyl group with release of ammonia. A majority of the Cys residues in proteins exists as cystine, which is a Cys dimer created by oxidation of thiol groups to form a disulfide cross-link.

Proline is a unique amino acid because it is the only *imino acid* in proteins. In proline, the propyl side chain is covalently linked to both the α-carbon atom and the α-amino group, forming a pyrrolidine ring structure.

5.2.1.4 Hydrophobicity of Amino Acids

One of the major factors affecting physicochemical properties, such as structure, solubility, fat-binding properties, and so forth, of proteins and peptides is the hydrophobicity of the constituent amino acid residues. Hydrophobicity can be defined as the excess free energy of a solute dissolved in water compared to that in an organic solvent under similar conditions. The most direct and simplest way to estimate hydrophobicities of amino acid side chains is experimental determination of free energy changes for dissolution of amino acid side chains in water and in an organic solvent, such as octanol or ethanol. The chemical potential of an amino acid dissolved in water can be expressed by:

$$\mu_{\mathrm{AA,w}} = \mu^{\circ}_{\mathrm{AA,w}} + RT \ln \gamma_{\mathrm{AA,w}} C_{\mathrm{AA,w}} \tag{5.6}$$

where $\mu^{\circ}_{\mathrm{AA,w}}$ is the standard chemical potential of the amino acid in the aqueous solution, γ_{AA} is the activity coefficient, C_{AA} is concentration, T is absolute temperature, and R is the gas constant. Similarly, the chemical potential of an amino acid dissolved in an organic solvent, for example, octanol, can be expressed as

$$\mu_{\mathrm{AA,oct}} = \mu^{\circ}_{\mathrm{AA,oct}} + RT \ln \gamma_{\mathrm{AA,oct}} C_{\mathrm{AA,oct}} \tag{5.7}$$

In saturated solutions, in which $C_{\mathrm{AA,w}}$ and $C_{\mathrm{AA,oct}}$ represent solubilities in water and octanol, respectively, the chemical potentials of the amino acid in water and octanol are the same, that is,

$$\mu_{\mathrm{AA,w}} = \mu_{\mathrm{AA,oct}} \tag{5.8}$$

Thus

$$\mu^{\circ}_{\mathrm{AA,oct}} + RT \ln \gamma_{\mathrm{AA,oct}} C_{\mathrm{AA,oct}} = \mu^{\circ}_{\mathrm{AA,w}} + RT \ln \gamma_{\mathrm{AA,w}} C_{\mathrm{AA,w}}. \tag{5.9}$$

The quantity $(\mu^{\circ}_{\mathrm{AA,w}} - \mu^{\circ}_{\mathrm{AA,oct}})$, which represents the difference between the standard chemical potentials arising from the interaction of the amino acid with octanol and with water, can be defined as the free energy change $(\Delta G^{0}_{\mathrm{tr,oct}\rightarrow\mathrm{w}})$ of transfer of the amino acid from octanol to water. Thus, assuming that the ratio of activity coefficients is one, the above equation can be expressed as

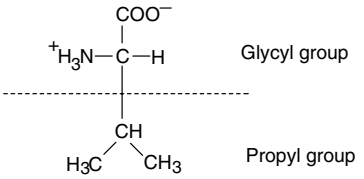
$$\Delta G^{0}_{\mathrm{tr,(oct}\rightarrow\mathrm{w)}} = -RT \ln(S_{\mathrm{AA,W}}/S_{\mathrm{AA,oct}}) \tag{5.10}$$

where $S_{\mathrm{AA,oct}}$ and $S_{\mathrm{AA,w}}$ represent solubilities of the amino acid in octanol and water, respectively.

As is true of all other thermodynamic parameters, ΔG^0_{tr} is an additive function. That is, if a molecule has two groups, A and B, covalently attached, the ΔG^0_{tr} for transfer from one solvent to another solvent is the sum of the free energy changes for transfer of groups A and B. That is,

$$\Delta G^0_{tr,AB} = \Delta G^0_{tr,A} + \Delta G^0_{tr,B} \tag{5.11}$$

The same logic can be applied to the transfer of an amino acid from octanol to water. For example, Val can be considered as a derivative of Gly with an isopropyl side chain at the α-carbon atom.



(5.12)

The free energy change of transfer of valine from octanol to water can then be considered as

$$\Delta G^0_{tr,Val} = \Delta G^0_{tr,Gly} + \Delta G^0_{tr,side\ chain} \tag{5.13}$$

or

$$\Delta G^0_{tr,side\ chain} = \Delta G^0_{tr,Val} - \Delta G^0_{tr,Gly} \tag{5.14}$$

In other words, the hydrophobicities of amino acid side chains can be determined by subtracting $\Delta G^0_{tr,Gly}$ from $\Delta G^0_{tr,Val}$.

The hydrophobicity values of amino acid side chains obtained in this manner are given in Table 5.3. Amino acid side chains with large positive ΔG^0_{tr} values are hydrophobic; they would prefer to be in an organic phase rather than in an aqueous phase. In proteins, these residues tend to locate themselves in the protein interior, where the polarity of the environment is similar to that of an organic phase. Amino acid residues with negative ΔG^0_{tr} values are hydrophilic, and these residues tend to locate themselves on the surface of protein molecules. The hydrophobicity of nonpolar residues is linearly correlated to their surface area, as shown in Figure 5.3.

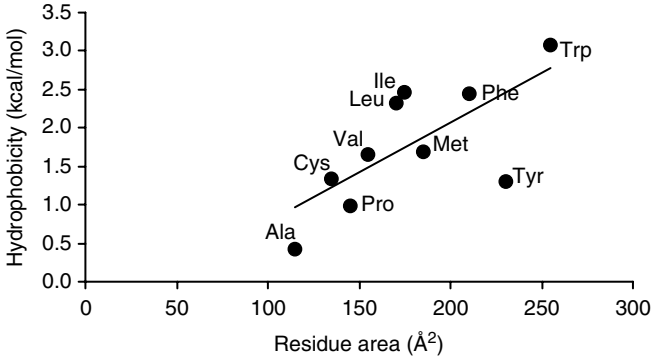


FIGURE 5.3 Correlation between surface area and hydrophobicity of nonpolar amino acid residues.

TABLE 5.4
Ultraviolet Absorbance and Fluorescence of Aromatic Amino Acids

Amino Acid	λ_{max} of Absorbance (nm)	Molar Extinction Coefficient (L mol^{-1} cm^{-1})	λ_{max} of Fluorescence (nm)
Phenylalanine	260	190	282[a]
Tryptophan	278	5500	348[b]
Tyrosine	275	1340	304[b]

[a] Excitation at 260 nm.
[b] Excitation at 280 nm.

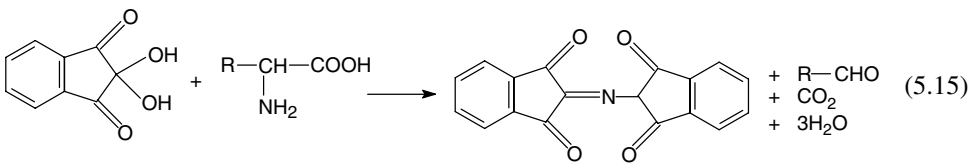
5.2.1.5 Optical Properties of Amino Acids

The aromatic amino acids Trp, Tyr, and Phe absorb light in the near-ultraviolet region (250–300 nm). In addition, Trp and Tyr also exhibit fluorescence in the ultraviolet region. The maximum wavelengths of absorption and fluorescence emission of the aromatic amino acids are given in Table 5.4. These amino acid residues are responsible for ultraviolet absorption properties of proteins in the 250–300 nm range, with maximum absorption at about 280 nm for most proteins. Since both absorption and fluorescence properties of these amino acids are influenced by the polarity of their environment, changes in the optical properties of proteins are often used as a means to monitor conformational changes in proteins.

5.2.2 Chemical Reactivity of Amino Acids

The reactive groups, such as amino, carboxyl, sulfhydryl, phenolic, hydroxyl, thioether (Met), imidazole, and guanyl, in free amino acids and proteins are capable of undergoing chemical reactions that are similar to those that would occur if they were attached to other small organic molecules. Typical reactions for various side-chain groups are presented in Table 5.5. Several of these reactions can be used to alter the hydrophilic and hydrophobic properties and the functional properties of proteins and peptides. Some of these reactions also can be used to quantify amino acids and specific amino acid residues in proteins. For example, reaction of amino acids with ninhydrin, *O*-phthaldialdehyde, or fluorescamine is regularly used in the quantification of amino acids.

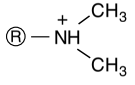
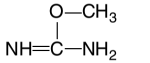
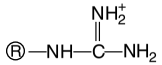
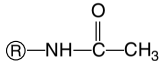
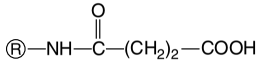
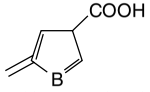
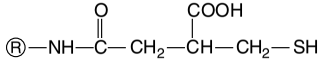
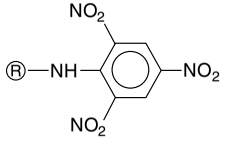
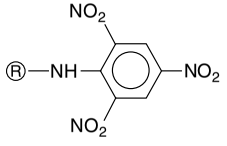
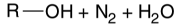
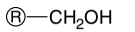
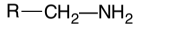
Reaction with ninhydrin: The ninhydrin reaction is often used to quantify free amino acids. When an amino acid is reacted with an excess amount of ninhydrin, one mole each of ammonia, aldehyde, CO_2, and hydrindantin are formed for every mole of amino acid consumed (Equation 5.15). The liberated ammonia subsequently reacts with one mole of ninhydrin and one mole of hydrindantin, forming a purple color product known as Ruhemann's purple, which has maximum absorbance at 570 nm. Proline and hydroxyproline give a yellow color product, which has maximum absorbance at 440 nm. These color reactions provide the basis for colorimetric determination of amino acids.



(5.15)

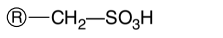
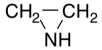
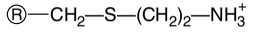
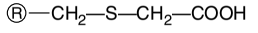
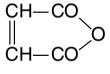
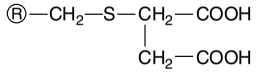
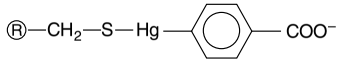
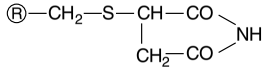
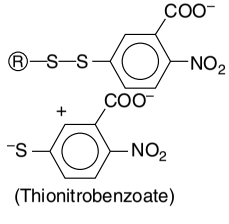
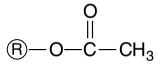
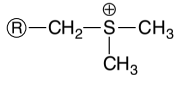
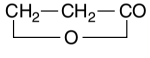
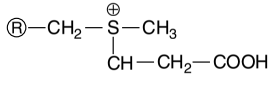
The ninhydrin reaction is usually used to determine the amino acid composition of proteins. In this case, the protein is first acid hydrolyzed to the amino acid level. The freed amino acids are then

TABLE 5.5
Chemical Reactions of Functional Groups in Amino Acids and Proteins

Type of Reaction	Reagent and Conditions	Product	Remarks
A. Amino groups			
1. Reductive alkylation	$HCHO$, $NaBH_4$ (formaldehyde)		Useful for radiolabeling proteins
2. Guanidation	 (*O*-methylisourea) pH 10.6, 4°C for 4 days		Converts lysyl side chain to homoarginine
3. Acetylation	Acetic anhydride		Eliminates the positive charge
4. Succinylation	Succinic anhydride		Introduces a negative charge at lysyl residues
5. Thiolation	 (Thioparaconic acid)		Eliminates positive charge and initiates thiol group at lysyl residues
6. Arylation	1-Fluoro-2,4-dinitrobenzene (FDNB)		Used for the determination of amino groups
	2,4,6-Trinitrobenzene sulfonic acid (TNBS)		The extinction coefficient is $1.1 \times 10^4\ M^{-1}\ cm^{-1}$ at 367 nm; used to determine reactive lysyl residues in proteins
7. Deamination	1.5 M $NaNO_2$ in acetic acid, 0°C		
B. Carboxyl groups			
1. Esterification	Acidic methanol		Hydrolysis of the ester occurs at pH > 6.0
2. Reduction	Borohydride in tetrahydrofuran, trifluoracetic acid		
3. Decarboxylation	Acid, alkali, heat treatment		Occurs only with amino acid, not with proteins

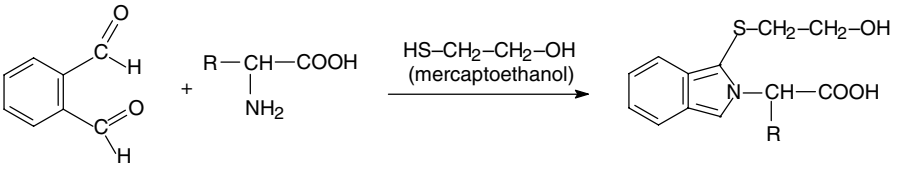
(Continued)

TABLE 5.5 (Continued)

Type of Reaction	Reagent and Conditions	Product	Remarks
C. Sulfhydryl group			
1. Oxidation	Performic acid		
2. Blocking	 (Ethyleneimine)		Introduces amino group
	Iodoacetic acid		Introduces one amino group
	 (Maleic anhydride)		Introduces two negative charges for each SH group blocked
	p-Mercuribenzoate		The extinction coefficient of this derivative at 250 nm (pH 7) is 7500 M^{-1} cm^{-1}; this reaction is used to determine SH content of proteins
	N-Ethylmaleimide		Used for blocking SH groups
	5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB)	 (Thionitrobenzoate)	One mole of thionitrobenzoate is released; the ϵ_{412} of thionitrobenzoate is 13,600 M^{-1} cm^{-1}; this reaction is used to determine SH groups in proteins
D. Serine and threonine			
1. Esterification	CH_3—COCl		
E. Methionine			
1. Alkyl halides	CH_3I		
2. β-Propiolactone			

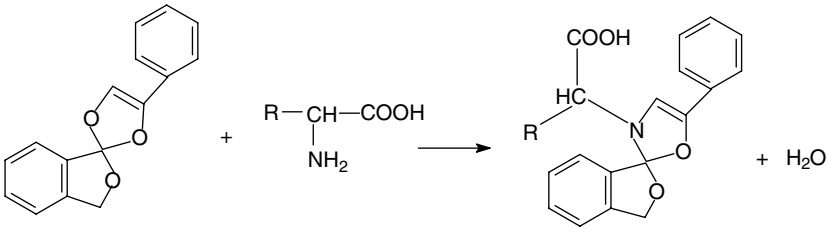
separated and identified using ion exchange/hydrophobic chromatography. The column eluates are reacted with ninhydrin and quantified by measuring absorbance at 570 and 440 nm.

Reaction with O-phthaldialdehyde: Reaction of amino acids with *O*-phthaldialdehyde (1,2-benzene dicarbonal) in the presence of 2-mercaptoethanol yields a highly fluorescent derivative that has an excitation maximum at 380 nm and a fluorescence emission maximum at 450 nm.



(5.16)

Reaction with fluorescamine: Reaction of amino acids, peptides, and proteins containing primary amines with fluorescamine yields a highly fluorescent derivative with fluorescence emission maximum at 475 nm when excited at 390 nm. This method can be used to quantify amino acids as well as proteins and peptides.



Fluorescamine

(5.17)

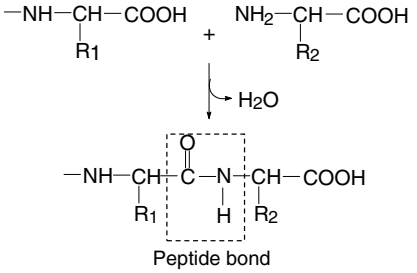
5.3 PROTEIN STRUCTURE

5.3.1 Structural Hierarchy in Proteins

Four levels of protein structure exist: *primary*, *secondary*, *tertiary*, and *quaternary*.

5.3.1.1 Primary Structure

The primary structure of a protein refers to the linear sequence in which the constituent amino acids are covalently linked through amide bonds, also known as peptide bonds. The peptide linkage results from condensation of the α-carboxyl group of ith amino acid and the α-amino group of $i+1$th amino acid with removal of a water molecule. In this linear sequence, all the amino acid residues are in the L-configuration. A protein with n amino acid residues contains $n-1$ peptide linkages.



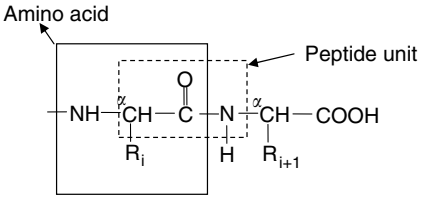
Peptide bond

(5.18)

The terminus with the free α-amino group is known as the N-terminal and that with the free α-COOH group is known as the C-terminal. By convention, N represents the beginning and C the end of the polypeptide chain when primary sequence information is indicated.

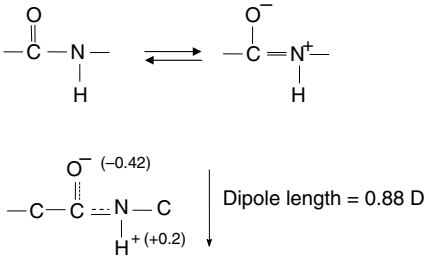
The chain length (n) and the sequence in which the n residues are linked determine the physicochemical, structural, biological properties, and functions of a protein. The amino acid sequence acts as the code for formation of secondary and tertiary structures and ultimately determines the protein's biological functionality. The molecular mass ranges from a few thousand Daltons (Da) to over a million Da. For example, titin, which is a single-chain protein found in muscle has, a molecular weight of over one million, whereas secretin has a molecular weight of about 2,300 Da. The molecular weight of most proteins is in the range of 20,000–100,000 Da.

The backbone of polypeptides can be depicted as repeating units of $-N-C-C^{\alpha}-$ or $-^{\alpha}C-C-N$. The expression $-NH-^{\alpha}CHR-CO-$ relates to an amino acid residue, whereas $-^{\alpha}CHR-CO-NH-$ represents a *peptide unit*.



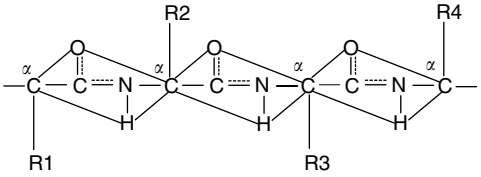
(5.19)

Although the CO—NH bond is depicted as a single covalent bond, in reality it has a partial double bond character because of the resonance structure caused by delocalization of electrons.



(5.20)

This has several important structural implications in proteins. First, the resonance structure precludes protonation of the peptide N—H group. Second, because of the partial double bond character, the rotation of the CO—NH bond is restricted to a maximum of 6°, known as ω-angle. Because of this restriction, each six-atom segment ($-C^{\alpha}-CO-NH-C^{\alpha}-$) of the peptide backbone lies in a single plane. The polypeptide backbone, in essence, can be depicted as a series of $-C^{\alpha}-CO-NH-C^{\alpha}-$ planes connected at the C_{α} atoms as shown below:

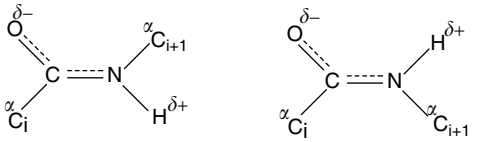


(5.21)

Since peptide bonds constitute about one-third of the total covalent bonds of the backbone, their restricted rotational freedom drastically reduces backbone flexibility. Only the $N-C^{\alpha}$ and $C^{\alpha}-C$ bonds have rotational freedoms, and these are termed ϕ (*phi*) and ψ (*psi*) dihedral angles, respectively.

These are also known as main-chain torsion angles. Third, delocalization of electrons also imparts a partial negative charge to the carbonyl oxygen atom and a partial positive charge to the hydrogen atom of the N—H group. Because of this, hydrogen bonding (*dipole–dipole interaction*) between the C═O and N—H groups of peptide backbone is possible under appropriate conditions.

Another consequence of the partial double-bond nature of the peptide bond is that the four atoms attached to the peptide bond can exist either in *cis* or *trans* configuration.



(5.22)

However, almost all protein peptide bonds exist in the *trans* configuration. This is due to the fact that the *trans* configuration is thermodynamically more stable than the *cis* configuration. Since *tran* → *cis* transformation increases the free energy of the peptide bond by 8.3 kcal/mol, isomerization of peptide bonds does not occur in proteins. One exception to this is peptide bonds involving proline residues. Since the free energy change for *trans* → *cis* transformation of peptide bonds involving proline residues is only about 1.86 kcal/mol, at high temperatures these peptide bonds sometimes do undergo *trans* → *cis* isomerization.

Although the N—C^{α} and C^{α}—C bonds are truly single bonds, and thus the N and P dihedral angles can theoretically have 360° rotational freedom, in reality their rotational freedoms are restricted by steric hindrances from side-chain atoms. These restrictions further decrease flexibility of the polypeptide chain.

5.3.1.2 Secondary Structure

Secondary structure refers to the periodic spatial arrangement of amino acid residues at certain segments of the polypeptide chain. The periodic structures arise when consecutive amino acid residues in a segment assume the same set of ϕ and ψ torsion angles. The twist of the ϕ and ψ angles is driven by near-neighbor or short-range noncovalent interactions between amino acid side chains, which leads to a decrease in local free energy. The *aperiodic* or *random* structure refers to those regions of the polypeptide chain where successive amino acid residues have different sets of ϕ and ψ torsion angles.

In general, two forms of periodic (regular) secondary structures are found in proteins. These are helical structures and extended sheet-like structures. The geometric characteristics of various regular structures found in proteins are given in Table 5.6.

Helical structures: Protein helical structures are formed when the ϕ and ψ angles of consecutive amino acid residues are twisted to a same set of values. By selecting different combinations of ϕ and ψ angles, it is theoretically possible to create several types of helical structures with different geometries. However, in proteins, only three types of helical structures, namely α-, 3_{10}-, and β-helix, are found.

Among the three helical structures, the α-helix is the major form found in proteins and it is the most stable (Figure 5.4). The pitch of this helix, that is, the increase in axial length per rotation, is 5.4 Å. Each helical rotation involves 3.6 amino acid residues, with each residue extending the axial length by 1.5 Å. The angle of rotation per residue is 100° (i.e., 360°/3.6). The amino acid side chains are oriented perpendicular to the axis of the helix.

α-Helix is stabilized by hydrogen bonding. In this structure, each backbone N–H group is hydrogen bonded to the C═O group of the fourth preceding residue. Thirteen backbone atoms

TABLE 5.6
Geometric Characteristics of Regular Polypeptide Conformations

Structure	ϕ	ψ	n	r	h (Å)	t
Right-handed α-helix	−58°	−47°	3.6	13	1.5	100°
π-Helix	−57°	−70°	4.4	16	1.15	81.8°
3_{10}-Helix	−49°	−26°	3	10	2	120°
Parallel β-sheet	−119°	+113°	2	—	3.2	—
Antiparallel β-sheet	−139°	+135°	2	—	3.4	—
Polyproline I (*cis*)	−83°	+158°	3.33	1.9	—	—
Polyproline II (*trans*)	−78°	+149°	3.00	3.12	—	—

ϕ and ψ represent dihedral angles of the N—C_α and C_α—C bonds, respectively; n is number of residues per turn; r, number of backbone atoms within a hydrogen bonded loop of helix; h, rise of helix per amino acid residue; $t = 360°/n$, twist of helix per residue.

Source: From Creighton, T. E. 1993. *Proteins: Structures and Molecular Properties*. W. H. Freeman Co., New York, pp. 158–159.

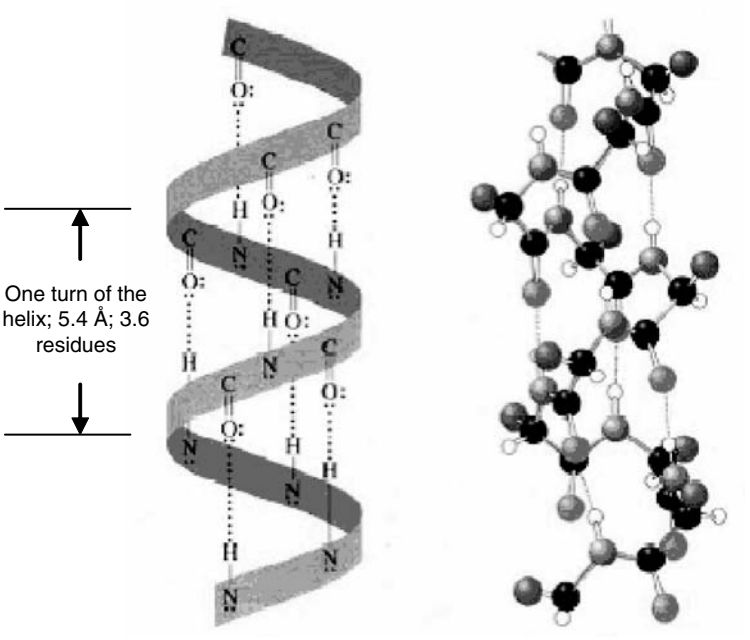


FIGURE 5.4 Spatial arrangement of polypeptides in α-helix. (From http://en.wikipedia.org/wiki/Alpha_helix)

are in this hydrogen-bonded loop, thus the α-helix is sometimes called the 3.6_{13} helix (Figure 5.4). The hydrogen bonds are oriented parallel to the helix axis, and the N, H, and O atoms of the hydrogen bond lie almost in a straight line, that is, the hydrogen bond angle is almost zero. The hydrogen bond length, that is, the N—H $\cdots$ O distance, is about 2.9 Å, and the strength of this bond is about 4.5 kcal/mol. The α-helix can exist in either a right- or left-handed orientation. However, the right-handed orientation is the more stable one.

The details for α-helix formation are embedded as a binary code in the amino acid sequence [61].The binary code is related to the arrangement of polar and nonpolar residues

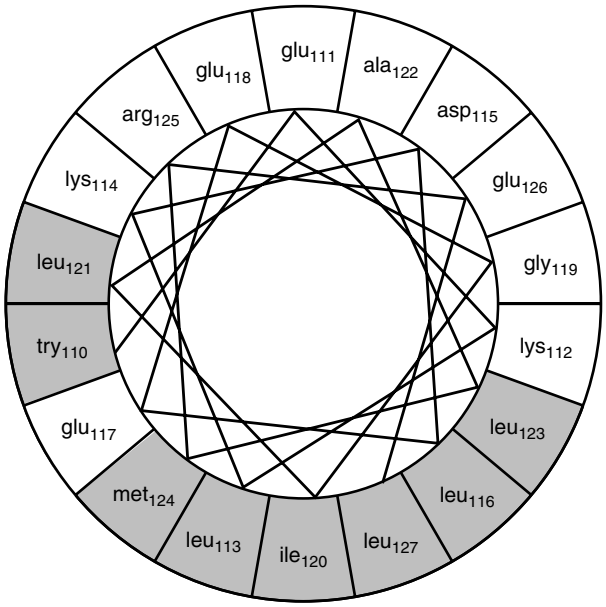


FIGURE 5.5 Cross-sectional view of the helical structure of residues 110–127 of bovine growth hormone. The top of the helical wheel (unfilled) represents the hydrophilic surface and the bottom (filled) represents the hydrophobic surface of the amphiphilic helix. (From Brems, D. N. 1990. In *Protein Folding* (Gierasch, L. M. and J. King, Eds.), American Association for the Advancement of Science, Washington, DC, p. 133. Courtesy of American Association for the Advancement of Science.)

in the sequence. Polypeptide segments with repeating seven amino acid (heptet) sequences of —P—N—P—P—N—N—P—, where P and N are polar and nonpolar residues, respectively, readily form α-helices in aqueous solutions. It is the binary code, and not the precise identities of the polar and nonpolar residues in the heptet sequence, that dictates α-helix formation. Slight variations in the binary code of the heptet are tolerated, provided other inter- or intramolecular interactions are favorable for α-helix formation. For example, tropomyosin, a muscle protein, exists entirely in a coiled-coil α-helical rod form. The repeating heptet sequence in this protein is —N—P—P—N—P—P—P—, which is slightly different from the above sequence. In spite of this variation, tropomyosin exists entirely in the α-helix form because of other stabilizing interactions in the coiled-coil rod [82].

Most of the α-helical structure found in proteins is amphiphilic in nature, that is, one-half of the helix's surface is occupied by hydrophobic residues and the other half by hydrophilic residues. This is schematically shown in the form of a helical wheel in Figure 5.5 [13]. In most proteins, the nonpolar surface of the helix faces the protein interior and is generally engaged in hydrophobic interactions with other nonpolar surfaces.

Other types of helical structures found in proteins are the β-helix and the 3_{10}-helix. The β- and 3_{10}-helices are about 0.5 kcal/mol and 1.0 kcal/mol, respectively, less stable than the α-helix. These helices exist only as short segments involving a few amino acid residues, and they are not major entities in most proteins.

In proline residues, because of the ring structure formed by covalent attachment of the propyl side chain to the amino group, rotation of the $N—C^{\alpha}$ bond is not possible, and therefore the ϕ angle has a fixed value of 70°. In addition, since there is no hydrogen at the nitrogen atom, it cannot form hydrogen bonds. Because of these two attributes, segments containing proline residues cannot form α-helices. In fact, proline is considered to be a α-helix breaker. Proteins containing high levels of proline residues tend to assume a random or aperiodic structure. For example, proline residues constitute about 17% of the total amino acid residues in β-casein, and 8.5% in α_{s1}-casein, and

because of the uniform distribution of these residues in their primary structures, α-helices are not present in these proteins and they have random structures. However, polyproline is able to form two types of helical structures, termed *polyproline I* and *polyproline II*. In polyproline I, the peptide bonds are in the *cis*-configuration, and in polyproline II they are in *trans*. Other geometric characteristics of these helices are given in Table 5.6. Collagen, which is the most abundant animal protein, exists as polyproline II-type helix. In collagen, on an average, every third residue is a glycine, which is preceded usually by a proline residue. Three polypeptide chains are entwined to form a triple helix, and the stability of the triple helix is maintained by interchain hydrogen bonds. This unique triple helix structure is responsible for the high tensile strength of collagen.

β-Sheet structure: The *β-sheet* is an extended structure with specific geometries given in Table 5.6. In this extended form, the C═O and N—H groups are oriented perpendicular to the direction of the chain, and therefore hydrogen bonding is possible only between segments (i.e., intersegment), and not within a segment (i.e., intrasegment). The β-strands are usually about 5–15 amino acid residues long. In proteins, two β-strands of the same molecule interact via hydrogen bonds, forming a sheet-like structure known as β-pleated sheet. In the sheet-like structure, the side chains are oriented perpendicular (above and below) to the plane of the sheet. Depending on the $N \rightarrow C$ directional orientations of the strands, two types of β-pleated sheet structures, namely *parallel β-sheet* and *antiparallel β-sheet*, can form (Figure 5.6). In parallel β-sheet the directions of the β-strands run parallel to each other, whereas in the other they run opposite to each other. These differences in chain directions affect the geometry of hydrogen bonds. In antiparallel β-sheets the N—H $\cdots$ O atoms lie in a straight line (zero H-bond angle), which enhances the stability of the hydrogen bond, whereas in parallel β-sheets they lie at an angle, which reduces the stability of the hydrogen bonds. Antiparallel β-sheets are, therefore, more stable than parallel β-sheets.

The binary code that specifies formation of β-sheet structures in proteins is —N—P—N—P—N—P—N—P—. Clearly, polypeptide segments containing alternating polar and nonpolar residues have

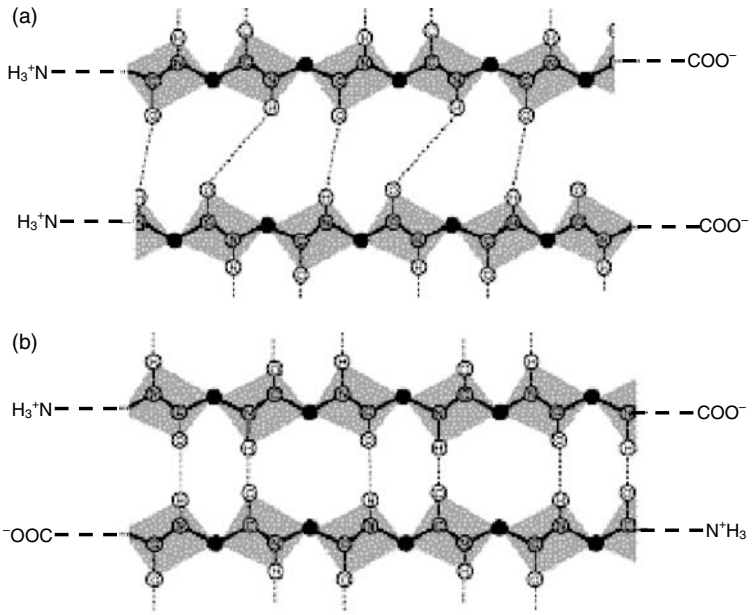


FIGURE 5.6 Parallel (a) and antiparallel (b) β-sheets. The dotted lines represent hydrogen bonds between peptide groups. The side chains at C_α atoms are oriented perpendicular (up or down) to the direction of the backbone. (From http://www.schoolscience.co.uk)

a high propensity to form β-sheet structures. Segments rich in bulky hydrophobic side chains, such as Val and Ile, also have a tendency to form a β-sheet structure. As expected, some variation in the code is tolerated.

The β-sheet structure is generally more stable than the α-helix. Proteins that contain large fractions of β-sheet structure usually exhibit high denaturation temperatures. Examples are β-lactoglobulin (51% β-sheet) and soy 11S globulin (64% β-sheet), which have thermal denaturation temperatures of 75.6 and 84.5°C, respectively. On the other hand, the denaturation temperature of bovine serum albumin, which has about 64% α-helix structure, is only about 64°C [25,27]. When solutions of α-helix-type proteins are heated and cooled, the α-helix is usually converted to β-sheet [27]. However, conversion from β-sheet to α-helix has not been observed in proteins.

Another common structural feature found in proteins is the β-bend or β-turn. This arises as a result of 180° reversal of the polypeptide chain involved in β-sheet formation. The hairpin-type bend is the result of antiparallel β-sheet formation, and the crossover bend is the result of parallel β-sheet formation. Usually, a β-bend involves a four-residue segment folding back on itself and the bend is stabilized by a hydrogen bond. The amino acid residues Asp, Cys, Asn, Gly, Tyr, and Pro are common in β-bends. The secondary structure contents of several proteins are given in Table 5.7.

5.3.1.3 Tertiary Structure

Tertiary structure refers to the spatial arrangement attained when a linear protein chain with secondary structure segments folds further into a compact three-dimensional form. The tertiary structures of β-lactoglobulin and phaseolin (the storage protein in kidney beans) are shown in Figure 5.7 [74,98].

TABLE 5.7
Secondary Structure Content of Selected Globular Proteins[a]

Protein	%α-Helix	%β-Sheet	%β-Turns	%Aperiodic
Deoxyhemoglobin	85.7	0	8.8	5.5
Bovine serum albumin	67.0	0	0	33.0
α_{s1}-Casein	15.0	12.0	19.0	54.0
β-Casein	12.0	14.0	17.0	57.0
κ-Casein	23.0	31.0	14.0	32.0
Chymotrypsinogen	11.0	49.4	21.2	18.4
Immunoglobulin G	2.5	67.2	17.8	12.5
Insulin (dimer)	60.8	14.7	10.8	15.7
Bovine trypsin inhibitor	25.9	44.8	8.8	20.5
Ribonuclease A	22.6	46.0	18.5	12.9
Egg lysozyme	45.7	19.4	22.5	12.4
Ovomucoid	26.0	46.0	10.0	18.0
Ovalbumin	49.0	13.0	14.0	24.0
Papain	27.8	29.2	24.5	18.5
α-Lactalbumin	26.0	14.0	0	60.0
β-Lactoglobulin	6.8	51.2	10.5	31.5
Soy 11S	8.5	64.5	0	27.0
Soy 7S	6.0	62.5	2.0	29.5
Phaseolin	10.5	50.5	11.5	27.5
Myoglobin	79.0	0	5.0	16.0

[a] The values represent % of total number of amino acid residues.

Source: Compiled from various sources.

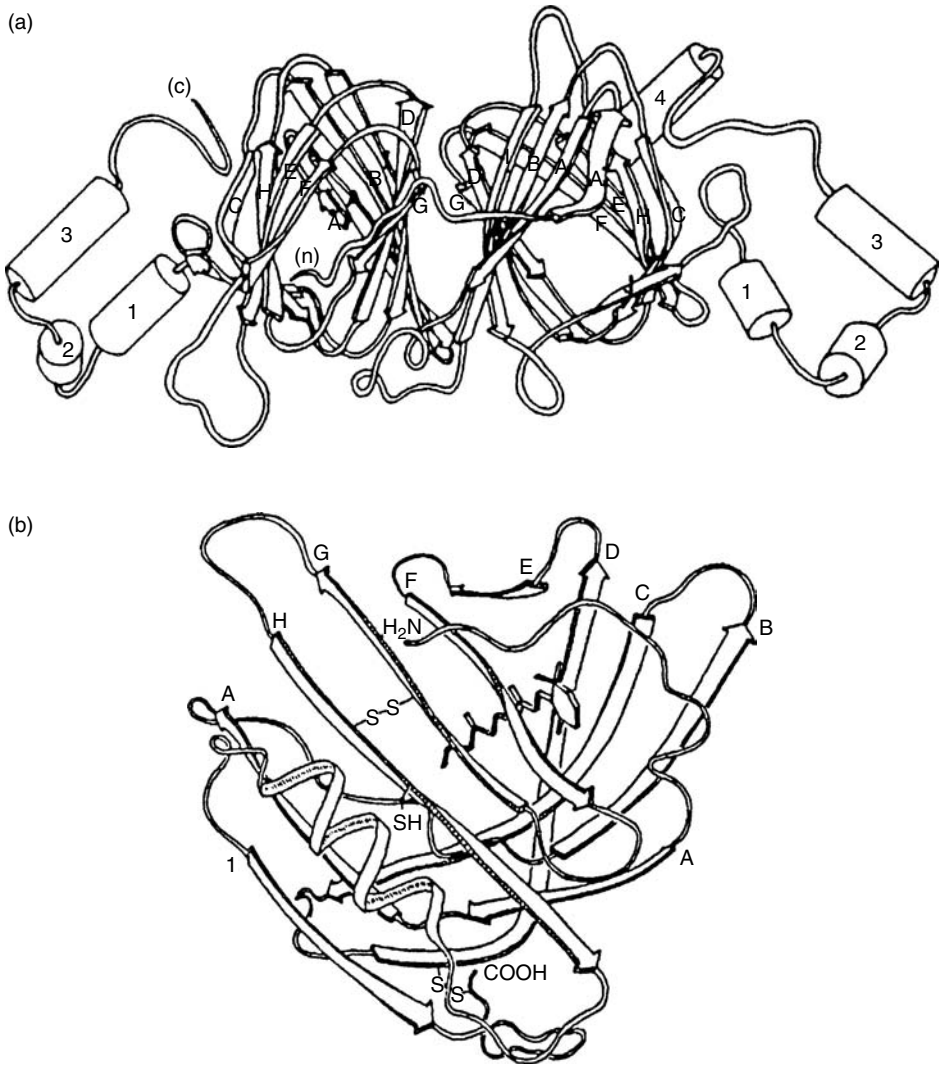


FIGURE 5.7 Tertiary structures of (a) phaseolin subunit and (b) β-lactoglobulin. The arrows indicate β-sheet strands and the cylinders indicate α-helix. (From Lawrence, M. C. et al. 1990. *EMBO J.* **9**:9–15 and Papiz, M. Z. et al. 1986. *Nature* **324**:383–385, respectively.)

Transformation of a protein from a linear configuration (primary structure) into a folded tertiary structure is a complex process. At the molecular level, the details for formation of a protein tertiary structure are present in its amino acid sequence. From a thermodynamic viewpoint, formation of tertiary structure involves optimization of various interactions (hydrophobic, electrostatic, van der Waals, and hydrogen bonding) between various groups in protein and the conformational entropy of the polypeptide chain, so that the net free energy of the molecule is reduced to the minimum value possible. The most important rearrangement that accompanies the reduction in free energy during formation of tertiary structure is the relocation of most of the hydrophobic residues at the interior of the protein structure away from the water environment and relocation of most of the hydrophilic residues, especially charged residues, at the protein–water interface. Although there is a strong general tendency for hydrophobic residues to be buried in the protein interior, this often can be accomplished only partially because of steric constraints. In fact, in most globular proteins, nonpolar residues occupy about 40–50% of the water accessible surface of protein molecules [84]. Also, some

polar groups are inevitably buried in the interior of proteins; however, these buried polar groups are invariably hydrogen bonded to other polar groups, such that their free energies are minimized in the apolar environment of the protein interior. The ratio of apolar and polar surfaces on a protein's surface enormously influences several of its physicochemical properties.

The folding of a protein from a linear structure to a folded tertiary structure is accompanied by a reduction in protein–water interfacial area. In fact, protein is forced to fold in order to mimize the protein–water interfacial area. The *accessible interfacial area* of a protein is defined as the total interfacial area of a three-dimensional space, occupied by the protein, as determined by figuratively rolling a spherical water molecule of radius 1.4 Å over the entire surface of the protein molecule. For native globular proteins, the accessible interfacial area (in $Å^2$) is a simple function of their molecular weight, M, as given by [84]:

$$A_s = 6.3M^{0.73}. \tag{5.23}$$

The total accessible interfacial area of a nascent polypeptide in its extended state (i.e., fully stretched molecule with no secondary, tertiary, or quaternary structure) is also correlated to its molecular weight by [84]:

$$A_t = 1.48M + 21 \tag{5.24}$$

The initial area of a protein that has folded during formation of a globular tertiary structure (i.e., A_b, buried area) can be estimated from Equations 5.23 and 5.24.

The fraction and distribution of hydrophilic and hydrophobic residues in the primary structure affects several physicochemical properties of the protein. For instance, the shape of a protein molecule is dictated by its amino acid sequence. If a protein contains a large number of hydrophilic residues distributed uniformly in its sequence, it will assume an elongated or rod-like shape. This is because, for a given mass, an elongated shape has a large surface-area-to-volume ratio so that more hydrophilic residues can be placed on the surface. On the other hand, if a protein contains a large number of hydrophobic residues, it will assume a globular (roughly spherical) shape. This minimizes the surface-area-to-volume ratio, enabling more hydrophobic residues to be buried in the protein interior. Among globular proteins, it is generally found that larger molecules contain larger fractions of nonpolar amino acids than do smaller molecules.

The tertiary structures of several single polypeptide proteins are made up of domains. Domains are defined as those regions of the polypeptide sequence that fold up into a tertiary form independently. These are, in essence, miniproteins within a single protein. The structural stability of each domain is largely independent of the others. In most single-chain proteins, the domains fold independently and then interact with each other to form the unique tertiary structure of the protein. In some proteins, as in the case of phaseolin (Figure 5.7), the tertiary structure may contain two or more distinct domains (structural entities) connected by a segment of the polypeptide chain. The number of domains in a protein usually depends on its molecular weight. Small proteins (e.g., lysozyme, β-lactoglobulin, and α-lactalbumin) with 100–150 amino acid residues usually form a single domain tertiary structure. Large proteins, such as immunoglobulin, contain multiple domains. The light chain of immunoglobulin G contains two domains, and the heavy chain contains four domains. The size of each of these domains is about 120 amino acid residues. Human serum albumin, which is made up of 585 amino acid residues, has three homologous domains, and each domain contains two subdomains [56].

5.3.1.4 Quaternary Structure

Quaternary structure refers to the spatial arrangement of a protein when it contains more than one polypeptide chain. Several biologically important proteins exist as dimers, trimers, tetramers, and so forth. Any of these quaternary complexes (also referred to as oligomers) can be made up of protein

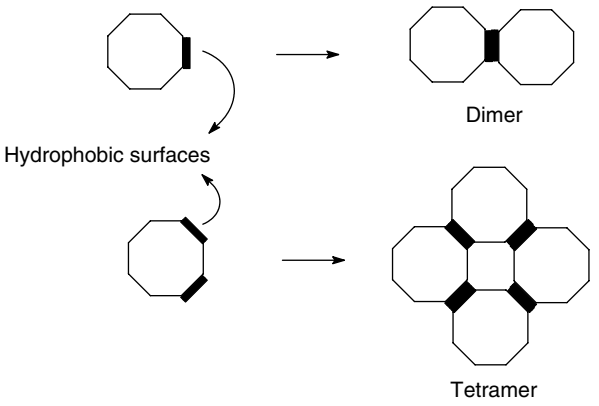


FIGURE 5.8 Schematic representation of formation of dimers and oligomers in proteins.

subunits (monomers) that are the same (homogeneous) or different (heterogeneous). For example, β-lactoglobulin exits as a dimer in the pH range 5–8, as an octomer in the pH range 3–5, and as a monomer above pH 8, and the monomeric units of these complexes are identical. On the other hand, hemoglobin is a tetramer made up of two different polypeptide chains, that is, α and β chains.

Formation of oligomeric structures is the result of specific protein–protein interactions. These are primarily driven by noncovalent interactions such as hydrogen bonding, hydrophobic and electrostatic interactions. The fraction of hydrophobic amino acids appears to influence the tendency to form oligomeric proteins. Proteins that contain >30% hydrophobic amino acid residues exhibit a greater tendency to form oligomeric structures than do those that contain fewer hydrophobic amino acid residues.

Formation of quaternary structure is primarily driven by the thermodynamic requirement to bury exposed hydrophobic surfaces of subunits. When the hydrophobic amino acid content of a protein is >30%, it is physically impossible to form a tertiary structure that will bury all of the nonpolar residues. Consequently, there is a greater likelihood of hydrophobic patches to exist on the surface, and interaction of these patches between adjacent monomers can lead to the formation of dimers, trimers, and so forth (Figure 5.8).

Many food proteins, especially cereal proteins, exist as oligomers of different polypeptides. As would be expected, these proteins typically contain more than 35% hydrophobic amino acid residues (Ile, Leu, Trp, Tyr, Val, Phe, and Pro). In addition, they also contain 6–12% proline [15]. As a consequence, cereal proteins exist in complex oligomeric structures. The major storage proteins of soybean, namely β-conglycinin and glycinin, contain about 41 and 39% hydrophobic amino acid residues, respectively. β-Conglycinin is a trimeric protein made up of three different subunits, and it exhibits complex association–dissociation phenomenon as a function of ionic strength and pH [89,123]. Glycinin is made up of 12 subunits, six of the subunits being acidic and the others basic. Each basic subunit is cross-linked to an acid subunit via a disulfide bond. The six acidic–basic pairs are held together in the oligomeric state by noncovalent interactions. Glycinin also exhibits complex association–dissociation behavior as a function of ionic strength [89].

In oligomeric proteins, the accessible surface area, A_s, is correlated to the molecular weight of the oligomer [84] by:

$$A_s = 5.3M^{0.76} \tag{5.25}$$

This relationship is different from that which applies to monomeric proteins. The surface area buried when the native oligomeric structure is formed from its constituent polypeptide subunits can

be estimated from the equation:

$$A_b = A_t - A_s = (1.48M + 21) - 5.3M_{0.76} \tag{5.26}$$

where A_t is the total accessible area of the nascent polypeptide subunits in their fully extended state.

5.3.2 Forces Involved in the Stability of Protein Structure

The process of folding of a random polypeptide chain into a unique three-dimensional structure is quite complex. As mentioned earlier, the basis for the biologically native conformation is encoded in the amino acid sequence of the protein. In the 1960s, Anfinsen and coworkers showed that when denatured ribonuclease was added to a physiological buffer solution, it refolded to its native conformation and regained almost 100% of its biological activity. Several enzymes have been subsequently shown to exhibit similar propensity. The slow but spontaneous transformation of an unfolded state to a folded state is facilitated by several intramolecular noncovalent interactions. The native conformation of a protein is a thermodynamic state in which various favorable interactions are maximized, and the unfavorable ones are minimized such that the overall free energy of the protein molecule is at the lowest possible value. The forces that contribute to protein folding may be grouped into two categories: (1) intramolecular interactions emanating from forces intrinsic to the protein molecule and (2) intramolecular interactions affected by the surrounding solvent. van der Waals and steric interactions belong to the former and hydrogen bonding, electrostatic, and hydrophobic interactions belong to the latter.

5.3.2.1 Steric Strains

Although the ϕ and ψ angles theoretically have 360° rotational freedom, their values are very much restricted because of steric hindrance from side-chain atoms. Because of this, segments of a polypeptide chain can assume only a limited number of configurations. Distortions in the planar geometry of the peptide unit, or stretching and bending of bonds, will cause an increase in the free energy of the molecule. Therefore, folding of the polypeptide chain can occur only in such a way that deformation of bond lengths and bond angles are avoided.

5.3.2.2 van der Waals Interactions

These are dipole-induced dipole and induced dipole-induced dipole interactions between neutral atoms in protein molecules. When two atoms approach each other, each atom induces a dipole in the other via polarization of the electron cloud. The interaction between these induced dipoles has an attractive as well as a repulsive component. The magnitudes of these forces are dependent on the interatomic distance. The attractive energy is inversely proportional to the sixth power of the interatomic distance, and the repulsive interaction is inversely proportional to the 12^{th} power of this distance. Therefore, at a distance r, the net interaction energy between two atoms is given by the potential energy function:

$$E_{vdW} = E_a + E_r = \frac{A}{r^6} + \frac{B}{r^{12}} \tag{5.27}$$

where A and B are constants for a given pair of atoms, and E_a and E_r are the attractive and repulsive interaction energies, respectively. van der Waals interactions are very weak, decrease rapidly with distance and become negligible beyond 6 Å. The van der Waals interaction energy for various pairs of atoms ranges from −0.04 to −0.19 kcal/mol. In proteins, however, since numerous pairs of atoms are involved in van der Waals interactions, sum of its contribution to protein folding and stability is very significant.

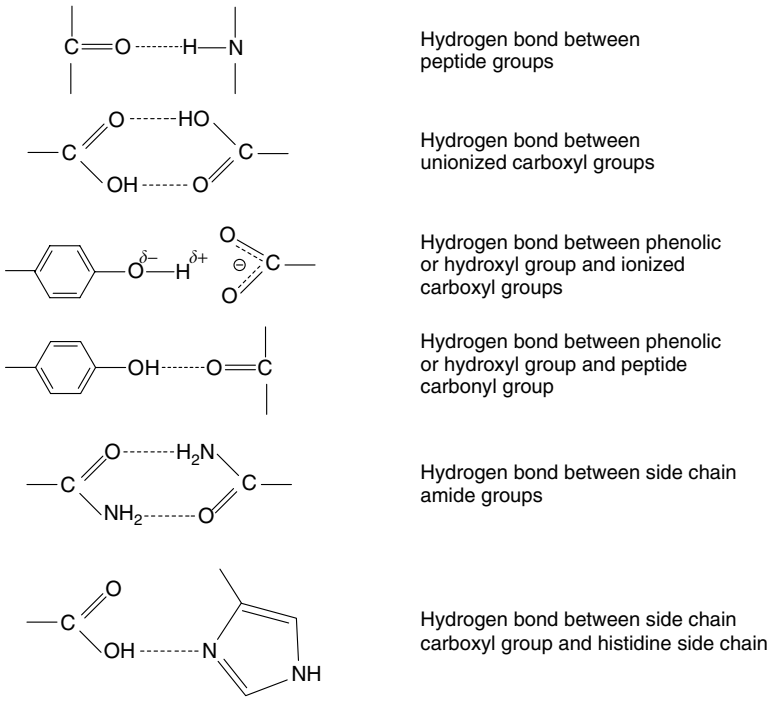


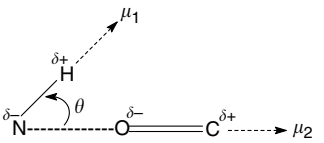
FIGURE 5.9 H-bonding groups in proteins. (From Scheraga, H. A. 1963. In *The Proteins*, 2nd edn., Vol. 1 (Neurath, H., Ed.), Academic Press, New York, pp. 478–594.)

5.3.2.3 Hydrogen Bonds

The hydrogen bond involves the interaction of a hydrogen atom that is covalently attached to an electronegative atom (such as N, O, or S) with another electronegative atom. Schematically, a hydrogen bond may be represented as D—H $\cdots$ A, where D and A are, the donor and acceptor electronegative atoms, respectively. The strength of a hydrogen bond ranges between 2 and 7.9 kcal/mol, depending on the pair of electronegative atoms involved and the bond angle.

Proteins contain several groups capable of forming hydrogen bonds. Some of the possible candidates are shown in Figure 5.9 [113]. Among these groups, the greatest number of hydrogen bonds are formed between the N—H and C═O groups of the peptide bonds in α-helix and β-sheet structures.

The peptide hydrogen bond can be considered as a strong permanent dipole–dipole interaction between the $N^{\delta-}$—$H^{\delta+}$ and $C^{\delta+}$═$O^{\delta-}$ dipoles as shown below:



(5.28)

The strength of the hydrogen bond is given by the potential energy function:

$$E_{\text{H-bond}} = \frac{\mu_1 \mu_2}{4\pi \varepsilon_0 \varepsilon r^3} \cos\theta \tag{5.29}$$

where μ_1 and μ_2 are the dipole moments, ε_0 is the permittivity of the vacuum, ε is the dielectric constant of the medium, r is the distance between the electronegative atoms, and θ is the hydrogen

bond angle. The hydrogen bond energy is directly proportional to the product of the dipole moments and to the cosine of the bond angle, and is inversely proportional to the third power of the N···O distance and to the dielectric constant of the medium. The strength of the hydrogen bond reaches a maximum when θ is zero, and it is zero when θ is 90°. The hydrogen bonds in α-helix and antiparallel β-sheet structures have a θ value very close to zero; whereas those in parallel β-sheets have larger θ values. The optimum N···O distance for maximum hydrogen bond energy is 2.9 Å. At shorter distances, the electrostatic repulsive interaction between the $N^{\delta-}$ and $O^{\delta-}$ atoms causes a significant decrease in the strength of the hydrogen bond. At longer distances weak dipole–dipole interaction between the N—H and C═O groups decreases the strength of the hydrogen bond. The strength of N—H···O═C hydrogen bonds in the interior of proteins, where the dielectric constant is close to 1, is typically about 4.5 kcal/mol. The "strength" refers to the amount of energy needed to break the bond.

The existence of hydrogen bonds in proteins is well established. Since formation of each hydrogen bond decreases the free energy of the protein by about –4.5 kcal/mol, it is commonly believed that they may act not only as the driving force for protein folding but also may contribute enormously to the stability of the native structure. However, this is not a valid assumption. Because water can compete for hydrogen bonding with N—H and C═O groups in proteins, hydrogen bonding between these groups cannot occur spontaneously, nor can formation of N—H···O═C hydrogen bonds be the driving force for formation of α-helix and β-pleated sheets in proteins. The hydrogen bond is primarily an ionic interaction. Like other ionic interactions, its stability also depends upon the dielectric constant of the environment. The stability of hydrogen bonds in secondary structures is mainly due to a low dielectric created by interaction between nonpolar residues. These bulky side chains prevent access of water to the N—H···O═C hydrogen bonds. They are only stable as long as they are protected from water.

5.3.2.4 Electrostatic Interactions

As noted earlier, proteins contain several amino acid residues with ionizable groups. At neutral pH, Asp and Glu residues are negatively charged, and Lys, Arg, and His are positively charged. At alkaline pH, Cys and Tyr residues assume a negative charge.

Depending upon the relative number of negatively and positively charged residues, proteins assume either a net negative or a net positive charge at neutral pH. The pH at which the net charge is zero is called the *isoelectric pH* (pI). The isoelectric pH is different from the *isoionic point*. Isoionic point is the pH of the protein solution in the absence of electrolytes. The isoelectric pH of a protein can be estimated from its amino acid composition and the pK_a values of the ionizable groups using the Hendersen–Hasselbach equation (Equation 5.5).

With few exceptions, almost all charged groups in proteins are distributed on the surface of the protein molecule. Since at neutral pH proteins assume either a net positive or a net negative charge, one might expect that the net repulsive interaction between like-charges would destabilize protein structure. It is also reasonable to assume that attractive interactions between oppositely charged groups at certain critical locations might contribute to the stability of the protein structure. In reality, however, the strength of these repulsive and attractive forces is minimized in aqueous solutions because of the high permittivity of water. The electrostatic interaction energy between two fixed charges q_1 and q_2 separated by distance r is given by:

$$E_{ele} = \pm \frac{q_1 q_2}{4\pi \varepsilon_0 \varepsilon r} \tag{5.30}$$

In vacuum or air ($\varepsilon = 1$), the electrostatic interaction energy between two charges at a distance of 3 to 5 Å is about ±110 to ±66 kcal/mol. In water, however, this interaction energy is reduced to ±1.4 to ±0.84 kcal/mol, which is of the order of thermal energy of the protein molecule at 37°C. Therefore, the attractive and repulsive electrostatic interactions between charges located on the protein surface

do not contribute significantly to protein stability. However, charged groups partially buried in the protein interior, where the permittivity is lower than that of water, usually form salt bridges with strong interaction energy. Thus, the electrostatic interaction energy may range between ±0.84 and ±110 kcal/mol depending on the distance and the local permittivity.

Although electrostatic interactions may not act as the primary driving force for protein folding, the penchant of charged groups to remain exposed to the aqueous environment certainly would influence the folding pattern.

5.3.2.5 Hydrophobic Interactions

It should be obvious from the foregoing discussions that, in aqueous solutions, the hydrogen bonding and electrostatic interactions between various polar groups in a polypeptide chain do not possess sufficient energy to act as driving forces for protein folding. These polar interactions in proteins are not very stable in an aqueous environment and their stabilities depend on maintenance of an apolar environment. The major force driving protein folding comes from hydrophobic interactions among nonpolar groups.

In aqueous solutions, the hydrophobic interaction between nonpolar groups is the result of thermodynamically unfavorable interaction between water and nonpolar groups. When a hydrocarbon is dissolved in water, the standard free energy change (ΔG) is positive and the volume (ΔV) and enthalpy change (ΔH) are negative. Even though ΔH is negative, meaning that there is favorable interaction between water and the hydrocarbon, ΔG is positive. Since $\Delta G = \Delta H - T\Delta S$ (where T is the temperature and ΔS is the entropy change), the positive change in ΔG must result from a large negative change in entropy, which offsets the favorable change in ΔH. The decrease in entropy is caused by formation of a clathrate or cage-like water structure around the hydrocarbon. Because of the net positive change in ΔG, interaction between water and nonpolar groups is highly restricted. Consequently, in aqueous solutions, nonpolar groups tend to aggregate, so that the area of direct contact with water is minimized (see Chapter 2). This water structure-induced interaction between nonpolar groups in aqueous solutions is known as hydrophobic interaction. In proteins, hydrophobic interaction between nonpolar side chains of amino acid residues is the major reason that proteins fold into unique tertiary structures in which a majority of the nonpolar groups are removed from the aqueous environment.

Since the hydrophobic interaction is the antithesis of solution of nonpolar groups in water, ΔG for hydrophobic interaction is negative and, ΔV, ΔH, and ΔS are positive. Unlike other noncovalent interactions, hydrophobic interactions are endothermic; that is, hydrophobic interactions are stronger at high temperatures and weaker at low temperatures (opposite to that for hydrogen bonds). The variation of hydrophobic free energy with temperature usually follows a quadratic function,

$$\Delta G_{H\phi} = a + bT + cT^2 \tag{5.31}$$

where a, b, and c are constants, and T is absolute temperature.

The hydrophobic interaction energy between two spherical nonpolar molecules can be estimated from the potential energy equation [59]:

$$E_{H\phi} = -20\frac{R_1 R_2}{R_1 + R_2} e^{-D/D_0} \text{ kcal/mol} \tag{5.32}$$

where R_1 and R_2 are the radii of the nonpolar molecules, D is the distance in nm between the molecules, and D_0 is the decay length (1 nm). Unlike electrostatic, hydrogen bonding, and van der Waals interactions, which follow a power law relationship with distance between interacting groups, the hydrophobic interaction follows an exponential relationship with distance between interacting groups. Thus, it is effective over relatively long distances (e.g., 10 nm).

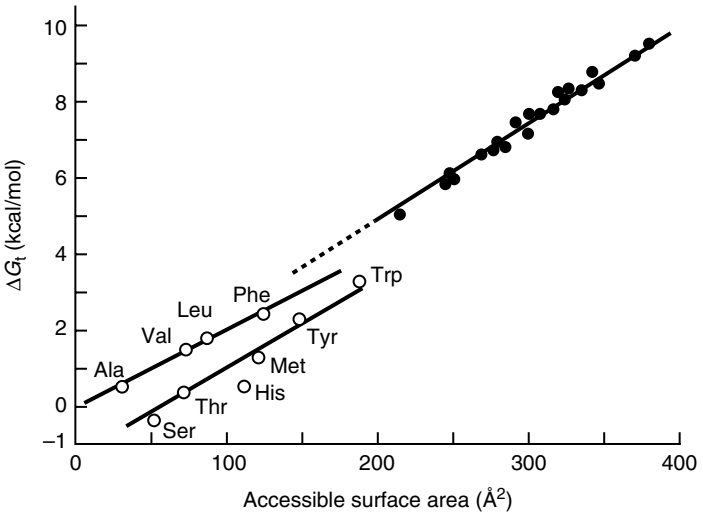


FIGURE 5.10 The relationship between hydrophobicity and accessible surface area of amino acid side chains (open circles) and hydrocarbons (filled circles). (From Richards, F. M. 1977. *Ann. Rev. Biophys. Bioeng.* **6**:151–176. Courtesy of Annual Reviews, Inc.)

The hydrophobic free energy of proteins cannot be quantified using the above equation because of involvement of several nonpolar groups. It is possible, however, to estimate the hydrophobic free energy of a protein using other empirical correlations. The hydrophobic free energy of a molecule is directly proportional to the nonpolar surface area that is accessible to water (Figure 5.10) [107]. The proportionality constant, that is, the slope, varies between 22 cal mol^{-1}Å^{-2} for Ala, Val, Leu, and Phe, and 26 cal mol^{-1}Å^{-2} for Ser, Thr, Trp, and Met side chains. On average, the hydrophobicity of amino acid side chains is about 24 cal mol^{-1}Å^{-2}. This is close to the 25 cal mol^{-1}Å^{-2} value for alkanes. This means that for the removal of every one Å^2 area of nonpolar surface from the water environment, a protein will decrease its free energy by about 24 cal/mol. Thus, the hydrophobic free energy of a protein can be estimated simply by multiplying the total buried surface area by 24 cal mol^{-1}Å^{-2}.

The buried surface area in several globular proteins and the estimated hydrophobic free energies are shown in Table 5.8 [11]. It is evident that hydrophobic free energy contributes significantly to the stability of protein structure. The average hydrophobic free energy per amino acid residue in globular proteins amounts to about 2.5 kcal/mol.

5.3.2.6 Disulfide Bonds

Disulfide bonds are the only covalent side chain cross-links found in proteins. They can occur both intramolecularly and intermolecularly. In monomeric proteins, disulfide bonds are formed as a result of protein folding. When two Cys residues are brought into proximity with proper orientation, oxidation of the sulfhydryl groups by molecular oxygen results in disulfide bond formation. Once formed, disulfide bonds help stabilize the folded structure of proteins.

Protein mixtures containing cystine and Cys residues are able to undergo sulfhydryl–disulfide interchange reactions as shown below:

(5.33)

TABLE 5.8
Accessible Surface Area (A_s), Buried Surface Area (A_b), and Hydrophobic Free Energy of Proteins

Protein	MW (Daltons)	A_s (Å^2)	A_b (Å^2)	$\Delta G_{H\phi}$ (kcal/mol)
Parvalbumin	11,450	5,930	11,037	269
Cytochrome c	11,930	5,570	12,107	294
Ribonuclease A	13,690	6,790	13,492	329
Lysozyme	14,700	6,620	15,157	369
Myoglobin	17,300	7,600	18,025	439
Retinol binding protein	20,050	9,160	20,535	500
Papain	23,270	9,140	25,320	617
Chymotrypsin	25,030	10,440	26,625	648
Subtilsin	27,540	10,390	30,390	739
Carbonic anhydrase B	28,370	11,020	30,988	755
Carboxypeptidase A	34,450	12,110	38,897	947
Thermolysin	34,500	12,650	38,431	935

A_s values are from Reference 84.
A_b was calculated from Equations 5.22 and 5.23.

This interchange reaction also can occur within a single denatured protein if it contains a free sulfhydryl group and a disulfide bond. The interchange reaction often leads to a decrease in stability of the protein molecule.

In summary, the formation of a unique three-dimensional protein structure is the net result of various repulsive and attractive noncovalent interactions and any covalent disulfide bonds.

5.3.3 Conformational Stability and Adaptability of Proteins

The stability of the native protein structure is defined as the difference in free energy between the native and denatured (or unfolded) states of the protein molecule. This is usually denoted as ΔG_D. This refers to the amount of energy needed to unfold a protein from the native state to the denatured state.

All of the noncovalent interactions discussed above, except the repulsive electrostatic interactions, contribute to the stability of the native protein structure. The stabilizing influence on the native structure of the total free energy changes attributed to these interactions amounts to hundreds of kcal/mol. However, the net ΔG_D of the majority of proteins is in the range of 5–20 kcal/mol. The major force that destabilizes the native structure is the conformational entropy of the polypeptide chain. The loss of translational, rotational, and vibrational motions that occurs when a polypeptide in a disordered state is folded into a compact structure decreases its conformational entropy. The increase in free energy resulting from this loss of conformational entropy is more than offset by favorable noncovalent interactions, resulting in a net decrease in free energy. Thus, the difference in free energy between the native and denatured states can be expressed as

$$\Delta G_{D\rightarrow N} = \Delta G_{\text{H-bond}} + \Delta G_{ele} + \Delta G_{H\phi} + \Delta G_{vdW} - T\Delta S_{conf} \tag{5.34}$$

where $\Delta G_{\text{H-bond}}$, ΔG_{ele}, $\Delta G_{H\phi}$, and ΔG_{vdW}, respectively, are free energy changes for hydrogen bonding, electrostatic, hydrophobic, van der Waals interactions, and ΔS_{conf} is the conformational entropy of the polypeptide chain. The ΔS_{conf} of a protein in the unfolded state is about 1.9 to 10 cal mol^{-1}K^{-1} per residue. Usually, an average value of 5.2 cal mol^{-1}K^{-1} per residue is assumed.

TABLE 5.9
ΔG_D Values for Selected Proteins

Protein	pH	T (°C)	ΔG_D (kcal/mol)
α-Lactalbumin	7	25	4.4
Bovine β-lactoglobulin A + B	7.2	25	7.6
Bovine β-lactoglobulin A	3.15	25	10.2
Bovine β-lactoglobulin B	3.15	25	11.9
T4 Lysozyme	3.0	37	4.6
Hen egg-white lysozyme	7.0	37	12.2
G-Actin	7.5	25	6.5
Lipase (from Aspergillus)	7.0	—	11.2
Troponin	7.0	37	4.7
Ovalbumin	7.0	25	6.0
Cytochrome C	5.0	37	7.9
Ribonuclease	7.0	37	8.1
α-Chymotrypsin	4.0	37	8.1
Trypsin	—	37	13.2
Pepsin	6.5	25	10.9
Growth hormone	8.0	25	14.2
Insulin	3.0	20	6.5
Alkaline phosphatase	7.5	30	20.3

ΔG_D represents $G_U - G_N$, where G_U and G_N are free energies of the denatured and native states, respectively, of a protein molecule.

Source: Compiled from several sources.

A protein with 100 amino acid residues at 310 K will have conformational entropy of about $5.2 \times 100 \times 310 = 161.2$ kcal/mol. This destabilizing conformational energy will reduce the net stability of the native structure resulting from noncovalent interactions.

The ΔG_D values, that is, energy required to unfold, of various proteins are presented in Table 5.9. These values clearly indicate that in spite of numerous intramolecular interactions, proteins are only marginally stable. For example, the ΔG_D values of most proteins correspond to an energy equivalent of one to three hydrogen bonds or about two to five hydrophobic interactions, suggesting that breakage of a few noncovalent interactions would destabilize the native structure of many proteins.

Conversely, it appears that proteins are not designed to be rigid molecules. They are flexible, their native state is in a metastable state, and breakage of one to three hydrogen bonds or a few hydrophobic interactions can easily cause a cooperative conformational change in proteins. Conformational adaptability to changing solution conditions is necessary to enable proteins to carry out several critical biological functions. For example, efficient binding of substrates or prosthetic ligands to enzymes invariably involves reorganization of polypeptide segments at the binding sites. On the other hand, proteins that require high structural stability to perform their physiological functions usually are stabilized by intramolecular disulfide bonds, which effectively counter the conformational entropy (i.e., the tendency of the polypeptide chain to unfold).

5.4 PROTEIN DENATURATION

The native structure of a protein is the net result of various attractive and repulsive interactions emanating from assorted intramolecular forces as well as interaction of various protein groups with surrounding solvent water. However, native structure is largely the product of the protein's environment. The native state is thermodynamically the most stable state with lowest feasible free

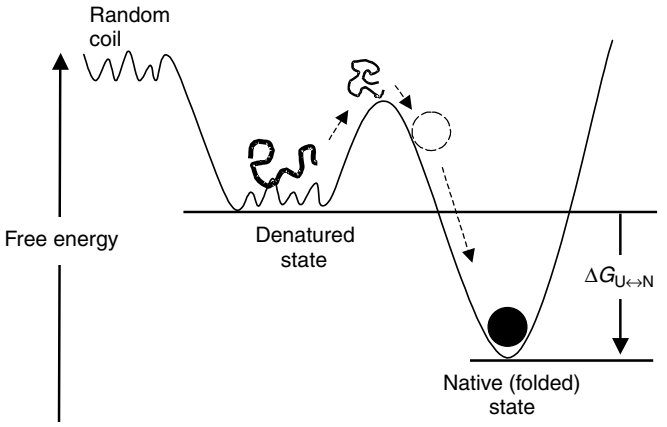


FIGURE 5.11 Schematic representation of the energy of a protein molecule as a function of its conformation. The conformation with the lowest energy is usually the native state.

energy. Any change in its environment, such as pH, ionic strength, temperature, solvent composition, and so forth, will force the molecule to assume a new equilibrium structure. Subtle changes in structure that do not drastically alter the molecular architecture of the protein are usually regarded as "conformational adaptability," whereas major changes in the secondary, tertiary, and quaternary structures without cleavage of backbone peptide bonds are regarded as "denaturation." From a structural standpoint, while the native structure of a protein is a well-defined entity with structural coordinates for each and every atom in the molecule obtainable from its crystallographic structure, it is not the case for the denatured state. Denaturation is a phenomenon wherein a well-defined initial state of a protein formed under physiological conditions is transformed into an ill-defined final state under nonphysiological conditions using a denaturing agent. It does not involve any chemical changes in the protein. In the denatured state, because of a greater degree of rotational motions of dihedral angles of the polypeptide chain, the protein can assume several conformational states differing only marginally in free energy. This is shown schematically in Figure 5.11. Some denatured states possess more residual folded structure than others. It should be noted that even in the fully denatured state, typical globular proteins, with the exception of gelatin, do not behave like a true random coil. This is because of the fact that the partial double bond character of the amide linkages and local steric restrictions caused by bulky side chains do not permit 360° rotational freedom for the polypeptide backbone.

The intrinsic viscosity ($[\eta]$) of a fully denatured protein is a function of the number of amino acid residues and is expressed by [121]:

$$[\eta] = 0.716\eta^{0.66} \tag{5.35}$$

where n is the number of amino acid residues in the protein.

Often, denaturation has a negative connotation, because it indicates loss of some properties. Many biologically active proteins lose their activity upon denaturation. In the case of food proteins, denaturation usually causes loss of solubility and some functional properties. However, from a food application standpoint, protein denaturation during processing is not always undesirable. In fact, in some cases it is highly desirable. For instance, partial denaturation of proteins at the air–water and oil–water interfaces improves their foaming and emulsifying properties, whereas excessive thermal denaturation of soy proteins diminishes their foaming and emulsifying properties. On the other hand, thermal denaturation markedly improves digestibility of legume proteins as a result of inactivation of trypsin inhibitors. In general, partially denatured proteins are more digestible than native proteins. In protein beverages, where high solubility and dispersibility of proteins is required, even partial

denaturation of protein during processing may cause flocculation and precipitation during storage and thus may adversely affect the sensory attributes of the product. Thermal denaturation is also a prerequisite for heat-induced gelation of food proteins. Thus, to develop appropriate processing strategies, it is imperative to have a basic understanding of the environmental and other factors that affect structural stability of proteins in food systems.

5.4.1 Thermodynamics of Denaturation

Denaturation is a phenomenon that involves transformation of a well-defined, folded structure of a protein, formed under physiological conditions, to an unfolded state under nonphysiological conditions. Since structure is not an easily quantifiable parameter, direct measurement of the fractions of native and denatured protein in a solution is not possible. However, conformational changes in proteins invariably affect several of its chemical and physical properties, such as ultraviolet-absorbance, fluorescence, viscosity, sedimentation coefficient, optical rotation, circular dichroism, reactivity of sulfhydryl groups, and enzyme activity. Thus, protein denaturation can be studied by monitoring changes in these physical and chemical properties.

When changes in a physical or chemical property, y, is monitored as a function of denaturant concentration or temperature, many monomeric globular proteins exhibit denaturation profiles as shown in Figure 5.12. y_N and y_D are y values for the native and denatured states, respectively, of the protein.

For most proteins, as denaturant concentration (or temperature) is increased, the value of y remains unchanged initially, and above a critical point its value changes abruptly from y_N to y_D within a narrow range of denaturant concentration or temperature. For a majority of globular proteins, this transition is very steep, indicating that protein denaturation is a cooperative process. That is, once a protein molecule begins to unfold, or once a few interactions in the protein are broken, the whole molecule completely unfolds with a further slight increase in denaturant concentration or temperature. This cooperative nature of unfolding suggests that globular proteins can exist only in the native and denatured states, that is, intermediate states are not possible. This is known as a

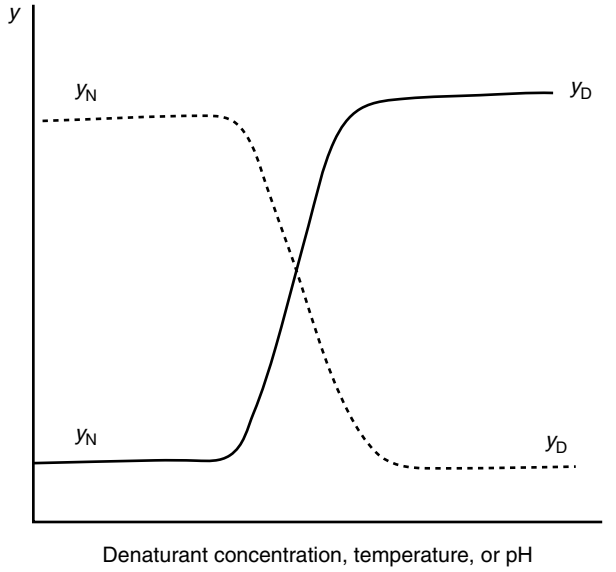


FIGURE 5.12 Typical protein denaturation curves. y represents any measurable physical or chemical property of the protein molecule that varies with protein conformation. y_N and y_D are the values of y for the native and denatured states, respectively.

"*two-state transition*" model. For this two-state model, the equilibrium between the native and the denatured state in the cooperative transition region can be expressed as

$$\begin{gathered} \mathrm{N} \overset{K_\mathrm{D}}{\rightleftharpoons} \mathrm{D} \\ K_\mathrm{D} = [\mathrm{D}]/[\mathrm{N}] \end{gathered} \tag{5.36}$$

where K_D is the equilibrium constant. Since the concentration of denatured protein molecules in the absence of a denaturant (or critical input of heat) is extremely low (about 1 in 10^9), estimation of K_D is not possible. However, in the transition region, that is, at sufficiently high denaturant concentration (or sufficiently high temperature), an increase in the population of the denatured protein molecule permits determination of the apparent equilibrium constant, $K_{\mathrm{D,app}}$. In the transition region, where both native and denatured protein molecules are present, the value of y is given by:

$$y = f_\mathrm{N} y_\mathrm{N} + f_\mathrm{D} y_\mathrm{D} \tag{5.37}$$

where f_N and f_D are the fractions of the protein in the native and denatured states, and y_N and y_D are y values for the native and denatured states, respectively. From Figure 5.12,

$$f_\mathrm{N} = \frac{(y_\mathrm{D} - y)}{(y_\mathrm{D} - y_\mathrm{N})} \tag{5.38}$$

$$f_\mathrm{D} = \frac{(y - y_\mathrm{N})}{(y_\mathrm{D} - y_\mathrm{N})} \tag{5.39}$$

The apparent equilibrium constant is given by:

$$K_\mathrm{app} = \frac{f_\mathrm{D}}{f_\mathrm{N}} = \frac{(y - y_\mathrm{N})}{(y - y_\mathrm{D})} \tag{5.40}$$

and the free energy of denaturation is given by:

$$\Delta G_\mathrm{app} = RT \ln K_{\mathrm{D,app}} \tag{5.41}$$

A plot of $-RT \ln K_{\mathrm{D,app}}$ vs. denaturant concentration is usually linear and thus the K_D and ΔG_D of the protein in pure water (or in buffer in the absence of denaturant) is obtained from the y-intercept. The enthalpy of denaturation, ΔH_D, is obtained from variation of the free energy change with temperature using the van't Hoff equation:

$$\Delta H_\mathrm{D} = -R \frac{\mathrm{d}(\ln K_\mathrm{D})}{\mathrm{d}(1/T)} \tag{5.42}$$

Monomeric proteins that contain two or more domains with different structural stabilities usually exhibit multiple transition steps in the denaturation profile. If the transition steps are well separated, the stabilities of each domain can be obtained from the transition profile by using the above two-state model. Denaturation of oligomeric proteins proceeds via dissociation of subunits, followed by denaturation of the subunits.

Protein denaturation can be reversible, especially for small monomeric proteins. When the denaturant is removed from the protein solution (or the sample is cooled), most monomeric proteins (in the absence of aggregation) refold to their native conformation under appropriate solution conditions, such as pH, ionic strength, redox potential, and protein concentration. Many proteins refold when the protein concentration is below 1 μM. Above 1 μM protein concentration, refolding

is partially inhibited because of greater intermolecular interaction at the cost of intramolecular interactions. A redox potential comparable to that of biological fluid facilitates formation of the correct pairs of disulfide bonds during refolding.

5.4.2 Denaturing Agents

5.4.2.1 Physical Agents

5.4.2.1.1 Temperature and Denaturation

Heat is the most commonly used denaturing agent in food processing and preservation. Proteins undergo varying degrees of denaturation during processing. This can affect their functional properties in foods and it is, therefore, important to understand the factors affecting protein denaturation.

When a protein solution is gradually heated above a critical temperature, it undergoes a sharp transition from the native state to the denatured state. The temperature at the transition midpoint, where the concentration ratio of native and denatured states is 1, is known either as the melting temperature T_m, or the denaturation temperature T_d. The mechanism of temperature-induced denaturation of proteins primarily involves the effect of temperature on the stability of noncovalent interactions. In this respect, the hydrogen bonding and electrostatic interactions, which are exothermic in nature, are destabilized, and hydrophobic interactions, which are endothermic, are stabilized as the temperature is increased. The strength of hydrophobic interactions reaches a maximum at about 70–80°C [22]. In addition to noncovalent interactions, temperature dependence of conformational entropy, $T\Delta S_{conf}$, also plays a major role in the stability of proteins. The conformational entropy of the chain increases as the temperature is increased, which favors an unfolded state. The net stability of a protein at a given temperature is then the sum total of these interactions. However, a careful analysis of the temperature effect on various interactions in proteins reveals the following: in globular proteins, the majority of charged groups exist on the surface of the protein molecule, fully exposed to the high dielectric aqueous medium. Because of the dielectric screening effect of water, attractive and repulsive electrostatic interactions between charged residues are greatly reduced. In addition, at physiological ionic strength, screening of charged groups in proteins by counter ions further reduces electrostatic interactions in proteins. Because of these facts, the influence of electrostatic interactions in proteins is not significant. Similarly, hydrogen bonds are unstable in an aqueous environment, and therefore their stability in proteins is dependent on hydrophobic interactions that create local low dielectric environment. This implies that so long as a nonpolar environment is maintained, the hydrogen bonds in proteins would remain intact when the temperature is increased. These facts suggest that although polar interactions are affected by temperature, they generally do not contribute to heat-induced denaturation of proteins. On the basis of these considerations, the stability of the native state of a protein can be simply regarded as the net free energy difference arising from hydrophobic interactions that tend to favor the folded state and the conformational entropy of the chain that favor the unfolded state. That is,

$$\Delta G_{N\rightarrow D} = \Delta G_{H\phi} + \Delta G_{conf} \tag{5.43}$$

The dependence of protein stability on temperature at constant pressure can be expressed as [33]

$$\frac{\partial \Delta G_{N\rightarrow D}}{\partial T} = \frac{\partial \Delta G_{H\phi}}{\partial T} + \frac{\partial \Delta G_{conf}}{\partial T} \tag{5.44}$$

Hydrophobic interactions are strengthened at higher temperatures; therefore, $\partial \Delta G_{H\phi}/\partial T > 0$. Conformational entropy increases upon unfolding of the protein; therefore, $\partial \Delta G_{conf}/\partial T < 0$. As the temperature is increased, the interplay between these opposing forces reach a point at which $\partial \Delta G_{N\rightarrow D}/\partial T \approx 0$. The temperature at which this occurs signifies the denaturation temperature (T_d)

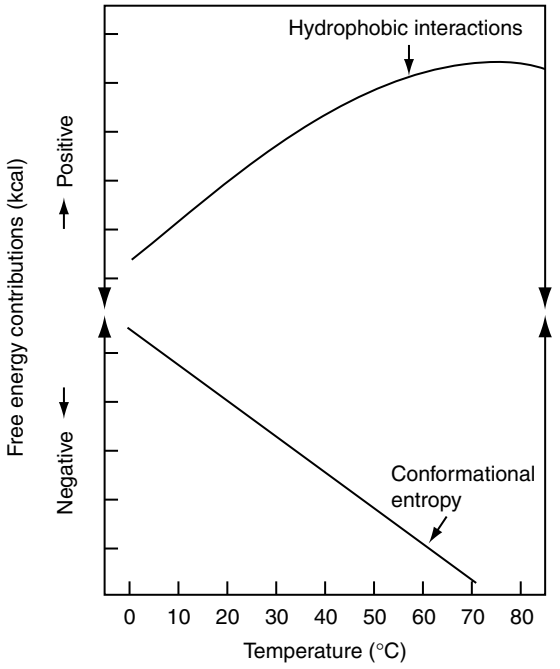


FIGURE 5.13 Relative changes in free energy contributions by hydrogen bonding, hydrophobic interactions, and conformational entropy to the stability of proteins as a function of temperature.

of the protein. The relative contributions of the major forces to stability of a protein molecule as a function of temperature are depicted in Figure 5.13. Note that the stability of hydrogen bonds in proteins is not significantly affected by the temperature. The T_d values of some proteins are listed in Table 5.10 [14].

It is often assumed that the lower the temperature, the greater will be the stability of a protein. This is not always true. Some proteins are denatured at cold temperatures [16]. For example (Figure 5.14) [18,73], the stability of lysozyme increases with lowering of temperature, whereas those of myoglobin and a mutant phage T4 lysozyme show maximum stability at about 30 and 12.5°C, respectively. Below and above these temperatures, myoglobin and phage T4 lysozyme are less stable. When stored below 0°C, these two proteins undergo cold-induced denaturation. The temperature of maximum stability (minimum free energy) depends on the relative inpact of temperature on the stabilizing and destabilizing forces in the protein. Proteins that are primarily stabilized by hydrophobic interactions are more stable at about ambient temperature than they are at refrigeration temperature. Intramolecular disulfide bonds in proteins tend to stabilize proteins at low as well as high temperatures because they counter conformational entropy of the protein chain.

Several food proteins undergo reversible dissociation and denaturation at low temperature. Glycinin, one of the storage proteins of soybean, aggregates and precipitates when stored at 2°C [68], then becomes soluble when returned to ambient temperature. When skim milk is stored at 4°C, β-casein dissociates from casein micelles, and this alters the physicochemical and rennetting properties of casein micelles. Several oligomeric enzymes, such as lactate dehydrogenase and glyceraldehyde-phosphate dehydrogenase, lose most of their enzyme activity when stored at 4°C; and this has been attributed to dissociation of the subunits. However, when warmed to and held at ambient temperature for a few hours, they reassociate and completely regain their activity [127].

The amino acid composition affects thermal stability of proteins. Proteins that contain a greater proportion of hydrophobic amino acid residues, especially Val, Ile, Leu, and Phe, tend to be more stable than the more hydrophilic proteins [137]. A strong positive correlation also exists between

TABLE 5.10
Thermal Denaturation Temperatures (T_d) and Mean Hydrophobicities of Proteins

Protein	T_d	Mean Hydrophobicity (kcal mol^{-1} $Residue^{-1}$)
Trypsinogen	55	0.89
Chymotrypsinogen	57	0.90
Elastase	57	—
Pepsinogen	60	0.97
Ribonuclease	62	0.78
Carboxypeptidase	63	—
Alcohol dehydrogenase	64	—
Bovine serum albumin	65	1.02
Hemoglobin	67	0.96
Lysozyme	72	0.90
Insulin	76	1.00
Egg albumin	76	0.97
Trypsin inhibitor	77	—
Myoglobin	79	1.05
α-Lactalbumin	83	1.03
Cytochrome C	83	1.06
β-Lactoglobulin	83	1.09
Avidin	85	0.92
Soy glycinin	92	—
Broadbean 11S protein	94	—
Sunflower 11S protein	95	—
Oat globulin	108	—

Source: Data were compiled from Bull, H. B. and K. Breese. 1973. *Arch. Biochem. Biophys*. **158**:681–686.

thermostability and the number percent of certain amino acid residues. For example, statistical analysis of fifteen different proteins has shown that thermal denaturation temperatures of these proteins are positively correlated ($r = .98$) to the sum of number percent of Asp, Cys, Glu, Lys, Leu, Arg, Trp, and Tyr residues. On the other hand, thermal denaturation temperatures of the same set of proteins are negatively correlated ($r = -.975$) to the sum of number percent of Ala, Asp, Gly, Gln, Ser, Thr, Val, and Tyr (Figure 5.15) [101]. Other amino acid residues have little influence on T_d.

Thermal stability of proteins from thermophilic and hyperthermophilic organisms, which can withstand extremely high temperatures, is also attributed to their unique amino acid composition [111]. These proteins contain lower levels of Asn and Gln residues than those from mesophilic organisms. The implication here is that because Asn and Gln are susceptible to deamidation at high temperatures, higher levels of these residues in mesophilic proteins may partly contribute to instability. The Cys, Met, and Trp contents, which can be oxidized easily at high temperatures, are also low in hyperthermostable proteins. On the other hand, thermostable proteins have high levels of Ile and Pro [117,126]. The high Ile content is believed to help in better packing of the interior core of the protein [110], which reduces buried cavities or void spaces. Absence of void spaces can reduce mobility of the polypeptide chain at high temperatures and this minimizes the increase in the configurational entropy of the polypeptide chain at high temperatures. A high content of Pro, especially in the loop regions of the protein chain, is believed to provide rigidity to the structure [75,87]. Examination of crystallographic structures of several proteins/enzymes from thermophilic

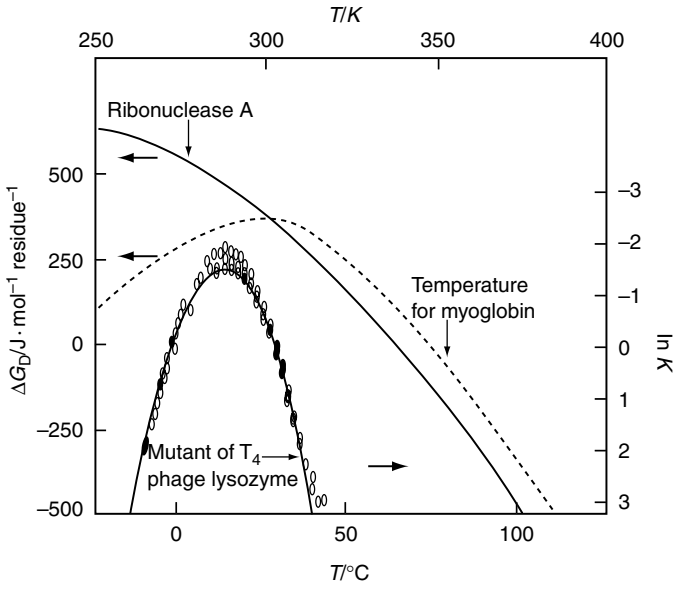


FIGURE 5.14 Variation of protein stability (ΔG_D) with temperature for myoglobin (- - - -), ribonuclease A (—), and a mutant of T4 phage lysozyme (-0-0-). K is the equilibrium constant. (Compiled from Chen, B. and J. A. Schellman. 1989. *Biochemistry* **28**:685–691 and Lapanje, S. 1978. *Physicochemical Aspects of Protein Denaturation*. Wiley-Interscience, New York.)

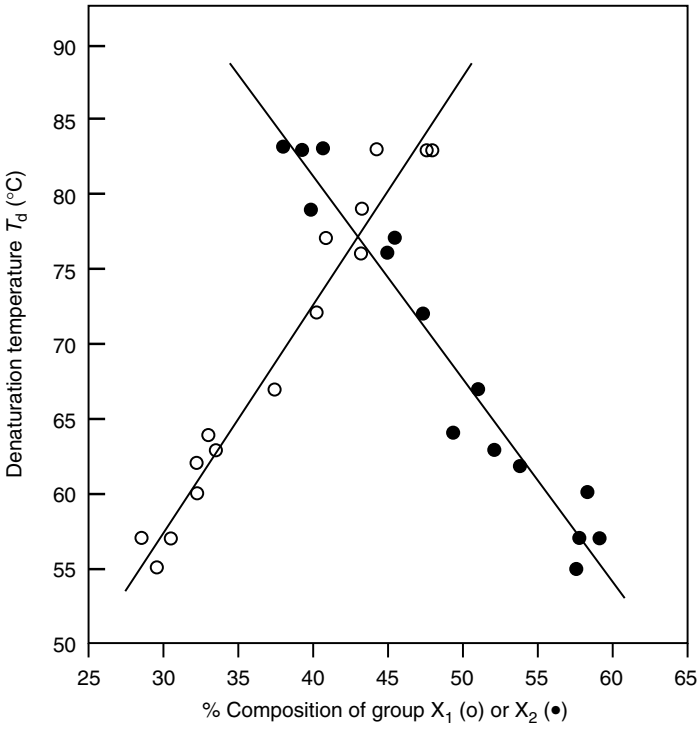


FIGURE 5.15 Group-correlations of amino acid residues to thermal stability of globular proteins. Group X_1 represents Asp, Cys, Glu, Lys, Leu, Arg, Trp, and Tyr. Group X_2 represents Ala, Asp, Gly, Gln, Ser, Thr, Val, and Tyr. (Adapted from Ponnuswamy, P. K. et al. 1982. *Int. J. Biol. Macromol*. **4**:186–190.)

organisms show that they also contain a significantly higher number of ion-pairs in crevices of proteins and a substantially higher amount of buried water molecules engaged in hydrogen bonding bridge between segments than in their mesophilic counterparts [4,132]. Taken together, it appears that polar interactions (both salt bridges and hydrogen bonding between segments) in the nonpolar protein interior are responsible for thermostability of proteins from thermophilic and hyperthermophilic organisms and such an environment is facilitated by a high content of Ile. As discussed earlier, it is conceivable that each salt bridge in the protein interior, where the dielectric constant is about 4, could increase the stability of protein structure by about 20 kcal/mol.

Thermal denaturation of monomeric globular proteins is mostly reversible. For example, when monomeric enzymes are heated above their denaturation temperatures, or even briefly held at 100°C, and then immediately cooled to room temperature, they fully regain their activities. However, thermal denaturation can become irreversible when the protein is heated at 90–100°C for a prolonged period even at neutral pH [5]. This irreversibility occurs because of several chemical changes in the protein, such as deamidation of Asn and Gln residues, cleavage of peptide bonds at Asp residues, destruction of Cys and cystine residues, and aggregation [5,124].

Water greatly facilitates thermal denaturation of proteins [46]. Dry protein powders are extremely stable to thermal denaturation. T_d decreases sharply as the water content is increased from 0 to 0.35 g water/g protein (Figure 5.16). An increase in water content from 0.35 to 0.75 g water/g protein causes only a marginal decrease in T_d. Above 0.75 g water/g protein, the T_d of the protein is same as in a dilute protein solution. The effect of hydration on thermostability is fundamentally related to protein dynamics. In the dry state, proteins have a static structure, that is, the polypeptide segments have restricted mobility. As the water content is increased, hydration and partial penetration of water into surface cavities causes swelling of the protein. This swollen state, where the protein and its water convert from an amorphous to a rubbery state, reaches a maximum value at water content of 0.3–0.4 g water/g protein at room temperature. The swelling of the protein increases chain mobility and flexibility, and the protein molecule assumes a more dynamic molten structure. When heated, this dynamic flexible structure provides greater access of water to salt bridges and peptide hydrogen bonds than is possible in the dry state, resulting in lower T_d.

Additives such as salts and sugars affect thermostability of proteins in aqueous solutions. Sugars such as sucrose, lactose, glucose, and glycerol stabilize proteins against thermal denaturation [69]. Addition of 0.5 M NaCl to proteins such as β-lactoglobulin, soy proteins, serum albumin, and oat globulin, significantly increases their T_d [25,27,54].

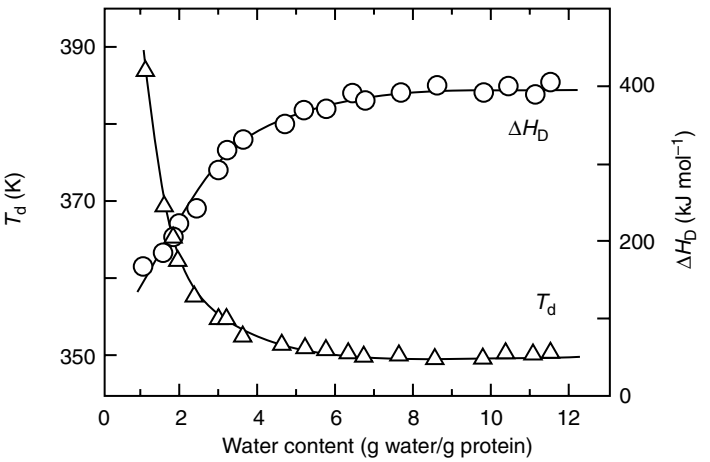


FIGURE 5.16 Influence of water content on the temperature (T_d) and enthalpy (ΔH_D) of denaturation of ovalbumin. (From Fujita, Y. and Y. Noda. 1981. *Bull. Chem. Soc. Japan* **54**:3233–3234.)

5.4.2.1.2 Hydrostatic Pressure and Denaturation

One of the thermodynamic variables that affect conformation of proteins is hydrostatic pressure. Unlike temperature-induced denaturation, which usually occurs in the range of 40–80°C at one atmospheric pressure, pressure-induced denaturation can occur at 25°C if the pressure is sufficiently great. Most proteins undergo pressure-induced denaturation in the range of 1–12 kbar as evidenced from changes in their spectral properties. The midpoint of pressure-induced transition occurs at 4–8 kbar [57].

Pressure-induced denaturation of proteins occurs mainly because proteins are flexible and compressible. Although amino acid residues are densely packed in the interior of globular proteins, some void spaces invariably exist and this leads to compressibility. The average partial specific volume of globular proteins in the hydrated state, υ^0, is about 0.74 mL/g. The partial specific volume can be considered as the sum of three components:

$$\upsilon^0 = V_C + V_{Cav} + \Delta V_{Sol} \tag{5.45}$$

where V_C is the sum of the atomic volumes, V_{Cav} is the sum of the volumes of the void spaces in the interior of the protein, and ΔV_{Sol} is the volume change due to hydration [47]. The larger the V_{Cav}, the larger is the contribution of void spaces to partial specific volume and the more unstable the protein will be when pressurized. Fibrous proteins are mostly devoid of void spaces, and hence they are more stable to hydrostatic pressure than globular proteins.

Pressure-induced denaturation of globular proteins is usually accompanied by a reduction in volume of about 30–100 mL/mol. This decrease in volume is caused by two factors: elimination of void spaces as the protein unfolds and hydration of the nonpolar amino acid residues that become exposed during unfolding. The latter event results in a decrease in volume (see Section 5.3.2). The volume change is related to the free energy change by the expression:

$$\Delta V = \frac{d(\Delta G)}{dp} \tag{5.46}$$

where p is the hydrostatic pressure.

If a globular protein completely unfolds during pressurization, the volume change should be about 2%. However, 30–100 mL/mol volume change observed in pressure-denatured proteins corresponds to only about 0.5% change in volume. This indicates that proteins only partially unfold even at hydrostatic pressure as high as 10 kbar.

Pressure-induced protein denaturation is highly reversible. Most enzymes, in dilute solutions, regain their activity once the pressure is decreased to atmospheric pressure [66]. However, regeneration of near complete activity usually takes several hours. In the case of pressure-denatured oligomeric proteins and enzymes, subunits first dissociate at 0.001–2 kbar, and then subunits denature at higher pressures [128]; when the pressure is removed, the subunits reassociate and almost complete restoration of enzyme activity occurs after several hours.

High hydrostatic pressures are being investigated as a food processing tool, for example, for microbial inactivation or gelation. Since high hydrostatic pressure (2–10 kbar) irreversibly damages cell membranes and causes dissociation of organelles in microorganisms, it will inactivate vegetative microorganisms [72]. Pressure gelation of egg white, 16% soy protein solution, or 3% actomyosin solution can be achieved by application of 1–7 kbar hydrostatic pressure for 30 min at 25°C. These pressure-induced gels are softer than thermally induced gels [94]. Also, exposure of beef muscle to 1–3 kbar hydrostatic pressure causes partial fragmentation of myofibrils, which may be useful as a means of tenderizing meat [119] and gelation of myofibrillar proteins [7]. Pressure processing, unlike thermal processing, does not harm essential amino acids, natural color and flavor, nor does it cause toxic compounds to develop. Thus, processing of foods with high hydrostatic pressure may prove advantageous (except for cost) for certain food products.

5.4.2.1.3 Shear and Denaturation

High mechanical shear generated by shaking, kneading, whipping, and so forth, can cause denaturation of proteins. Many proteins denature and precipitate when they are vigorously agitated [93]. In this circumstance, denaturation occurs because of incorporation of air bubbles and adsorption of protein molecules to the air–liquid interface. Since the energy of the air–liquid interface is greater than that of the bulk phase, proteins undergo conformational changes at the interface. The extent of conformational change depends on the flexibility of the protein. Highly flexible proteins denature more readily at an air–liquid interface than do rigid proteins. The nonpolar residues of denatured protein orient toward the gas phase and the polar residues orient toward the aqueous phase.

Several food processing operations involve high pressure, shear, and high temperature, for example, extrusion, high-speed blending, and homogenization. When a rotating blade produces a high shear rate, subsonic pulses are created and cavitation also occurs at the trailing edges of the blade. Both these events contribute to protein denaturation. The greater the shear rate, the greater is the degree of denaturation. The combination of high temperature and high shear force causes irreversible denaturation of proteins. For example, when a 10–20% whey protein solution at pH 3.5–4.5 and at 80–120°C is subjected to a shear rate of 7,500–10,000/s, it forms insoluble spherical macrocolloidal particles of about 1 μm diameter. A hydrated material produced under these conditions, "Simplesse," has a smooth, emulsion-like organoleptic character [118].

5.4.2.2 Chemical Agents

5.4.2.2.1 pH and Denaturation

Proteins are more stable against denaturation at their isoelectric point than at any other pH. At neutral pH, most proteins are negatively charged and a few are positively charged. Since the net electrostatic repulsive energy is small compared to other favorable interactions, most proteins are stable at around neutral pH. However, strong intramolecular electrostatic repulsion caused by high net charge at extreme pH values results in swelling and unfolding of the protein molecule. The degree of unfolding is greater at extreme alkaline pH values than it is at extreme acid pH values. The former behavior is attributed to ionization of partially buried carboxyl, phenolic, and sulfhydryl groups that cause unravelling of the polypeptide chain as they attempt to expose themselves to the aqueous environment. pH-induced denaturation is mostly reversible. However, in some cases, partial hydrolysis of peptide bonds, deamidation of Asn and Gln, destruction of sulfhydryl groups at alkaline pH, or aggregation can result in irreversible denaturation of proteins.

5.4.2.2.2 Organic Solvents and Denaturation

Organic solvents affect the stability of protein hydrophobic interactions, hydrogen bonding, and electrostatic interactions in different ways [52]. Since nonpolar side chains are more soluble in organic solvents than in water, organic solvents weaken hydrophobic interactions. On the other hand, since the stability and formation of peptide hydrogen bonds are enhanced in a low permittivity environment, certain organic solvents may actually strengthen or promote formation of peptide hydrogen bonds. For example, 2-chloroethanol causes an increase in α-helix content in globular proteins. The action of organic solvents on electrostatic interactions is twofold. By decreasing permittivity, they enhance electrostatic interactions between oppositely charged groups and also enhance repulsion between groups with like charge. The net effect of an organic solvent on protein structure, therefore, usually depends on the magnitude of its effect on various polar and nonpolar interactions. At low concentration, some organic solvents can stabilize several enzymes against denaturation [9]. At high concentrations, however, all organic solvents cause denaturation of proteins because of their solubilizing effect on nonpolar side chains.

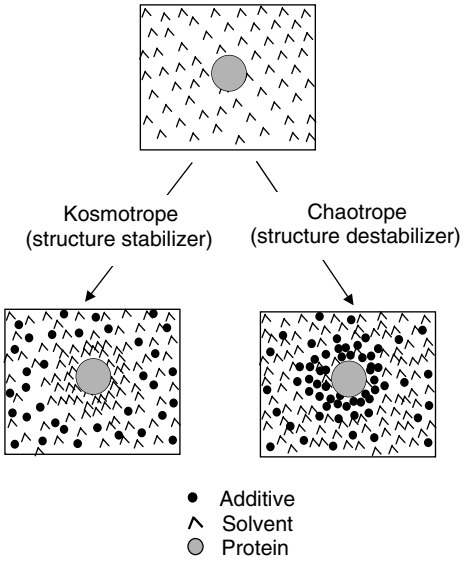


FIGURE 5.17 Schematic representation of preferential binding and preferential hydration of protein in the presence of additives. (Adapted from Creighton, T. E. 1993. *Proteins: Structures and Molecular Properties*. W. H. Freeman Co., New York, pp. 158–159.)

5.4.2.2.3 Denaturation by Small Molecular Weight Additives

Several small molecular weight solutes, such as urea, guanidine hydrochloride (GuHCl), detergents, sugars, and neutral salts affect protein stability in aqueous solutions. While urea, GuHCl, and detergents destabilize the native conformation of proteins [34], sugars tend to stabilize the native structure. In the case of neutral salts, while certain salts, such as sulfate, phosphate, and fluoride salts of sodium, termed as kosmotropes, stabilize protein structure, other salts, such as bromide, iodide, perchlorate, and thiocyanate, termed as chaotropes, destabilize protein structure.

The stabilizing or destabilizing effects of small molecular weight additives on proteins is believed to follow a universal mechanism. This is related to their preferential interaction with the aqueous phase and the protein surface. Additives that stabilize protein structure bind very weakly to the protein surface but enhance preferential hydration of the protein surface (Figure 5.17). Such additives are generally excluded from the region surrounding the protein; that is, their concentration near the protein is lower than in the bulk solution. This concentration gradient presumably creates an osmotic pressure gradient surrounding the protein molecule, sufficient enough to elevate the thermal denaturation temperature of the protein. For instance, studies on protein stabilization by glycerol have shown that lysozyme in glycerol solutions assumes a slightly compressed state compared with its state in water [51]. This might be due to creation of an exclusion zone around the protein surface for glycerol and development of an osmotic pressure gradient.

In the case of additives that destabilize protein structure, the opposite seems to be true. That is, those additives that decrease the stability of proteins preferentially bind to the protein surface and cause dehydration of the protein. In such cases, water molecules are excluded from the region surrounding the protein and the concentration of the additive in this water-excluded region is higher than in the bulk solvent. Favorable interaction of such additives with protein surface, particularly the nonpolar surface, promotes unfolding of the protein so that the buried nonpolar surfaces are further exposed for favorable interaction with the additive.

When a protein is exposed to a mixture of stabilizing and destabilizing solutes, the net effect on protein stability generally follows an additivity rule. For example, sucrose and polyols are considered to be protein structure stabilizers, whereas GuHCl is a structure destabilizer. When sucrose is mixed

with GuHCl, the concentration of GuHCl required for unfolding proteins increases with increase of sucrose concentration [122]. Furthermore, alterations in water structure caused by GuHCl and urea are countered by the addition of polyhydric compounds such as sucrose. Thus, in the presence of a polyol protein denaturation requires a higher GuHCl and urea concentration [122]. This also underscores that changes in water structure in the presence of additives is involved in some fundamental way in the transmission of the effects of additives on protein stability. The exact mechanism is still elusive, partly because "water structure" is not yet a well-defined concept.

5.4.2.2.4 Organic Solutes and Denaturation

Organic solutes, notably urea and GuHCl, cause denaturation of proteins. For many globular proteins, the midpoint of transition from the native to denatured state occurs at 4–6 M urea and at 3–4 M GuHCl at room temperature. Complete transition often occurs in 8 M urea and in about 6 M GuHCl. GuHCl is a more powerful denaturant than urea because of its ionic character. Many globular proteins do not undergo complete denaturation even in 8 M urea, whereas in 8 M GuHCl they usually exist in a random coil state (completely denatured).

Denaturation of proteins by urea and GuHCl is thought to involve two mechanisms. The first mechanism involves preferential binding of urea and GuHCl to the denatured protein. Removal of denatured protein as a protein-denaturant complex shifts the N $\longleftrightarrow$ D equilibrium to the right. As the denaturant concentration is increased, continuous conversion of the protein to protein-denaturant complex eventually results in complete denaturation of the protein. Since binding of denaturant to denatured protein is very weak, a high concentration of denaturant is needed to cause complete denaturation. The second mechanism involves solubilization of hydrophobic amino acid residues in urea and GuHCl solutions. Since urea and GuHCl have the potential to form hydrogen bonds, at high concentration these solutes breakdown the hydrogen-bonded structure of water. This destructuring of solvent water makes it a better solvent for nonpolar residues. This results in unfolding and solubilization of apolar residues from the interior of the protein molecule.

Urea- or GuHCl-induced denaturation is reversible. However, complete reversibility of urea-induced protein denaturation is sometimes difficult. This is because some urea converts to cyanate and ammonia. Cyanate reacts with amino groups and alters the charge of the protein.

5.4.2.2.5 Detergents and Denaturation

Detergents, such as sodium dodecyl sulfate (SDS), are powerful protein denaturing agents. SDS at 3–8 mM concentration denatures most globular proteins. The mechanism involves preferential binding of detergent to the denatured protein molecule. This causes a shift in equilibrium between the native and denatured states. Unlike urea and GuHCl, detergents bind strongly to denatured proteins and this is the reason for complete denaturation at a relatively low detergent concentration of 3–8 mM. Because of this strong binding, detergent-induced denaturation is irreversible. Globular proteins denatured by SDS do not exist in a random coil state; instead, they assume a α-helical rod shape in SDS solutions. This rod shape is properly regarded as denatured.

5.4.2.2.6 Chaotropic Salts and Denaturation

Salts affect protein stability in two different ways. At low concentrations, ions interact with proteins via nonspecific electrostatic interactions. This electrostatic neutralization of protein charges usually stabilizes protein structure. Complete charge neutralization by ions occurs at or below 0.2 M ionic strength and it is independent of the nature of the salt. However, at higher concentrations (>1 M), salts have ion specific effects that influence the structural stability of proteins. Salts such as Na_2SO_4 and NaF enhance, whereas NaSCN and $NaClO_4$ weaken it. Protein structure is influenced more by anions than by cations. For example, the effect of various sodium salts on the thermal denaturation temperature of β-lactoglobulin is shown in Figure 5.18. At equal ionic strength, Na_2SO_4 and NaCl increase T_d, whereas NaSCN and $NaClO_4$ decrease it. Regardless of their chemical make up and conformational differences, the structural stability of macromolecules, including DNA, is adversely

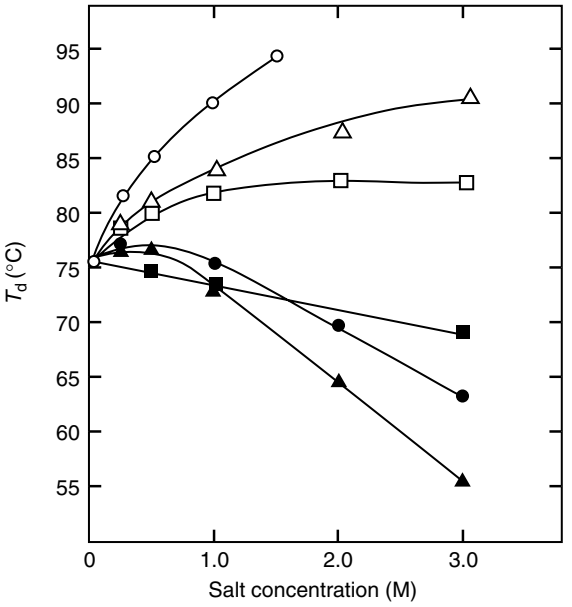


FIGURE 5.18 Effects of various sodium salts on the temperature of denaturation, T_d, of β-lactoglobulin at pH 7.0. △, NaCl; NaBr; ●, $NaClO_4$; ▲, NaSCN; ■, urea. (From Damodaran, S. 1989. *Int. J. Biol. Macromol.* **11**:2–8.)

affected by high concentrations of salts [21]. NaSCN and $NaClO_4$ are strong denaturants. The relative ability of various anions at isoionic strength to influence the structural stability of protein (and DNA) in general follows the series, $F^- < SO_4^{=} < Cl^- < Br^- < I^- < ClO_4^- < SCN^- < Cl_3CCOO^-$. This ranking is known as the Hofmeister series or chaotropic series. Floride, chloride, and sulfate salts are structure stabilizers, whereas the salts of other anions are structure destabilizers.

The mechanism of salts effects on the structural stability of proteins is related to their relative ability to bind to and alter hydration properties of proteins. Salts that stabilize proteins enhance hydration of proteins and bind weakly, whereas salts that destabilize proteins decrease protein hydration and bind strongly [8]. These effects are primarily the consequence of energy perturbations at the protein–water interface. On a more fundamental level, protein stabilization or denaturation by salts is related to their effect on bulk water structure. Salts that stabilize protein structure also enhance the hydrogen-bonded structure of water, and salts that denature proteins also breakdown bulk water structure and make it a better solvent for apolar molecules. In other words, the denaturing effect of chaotropic salts might be related to destabilization of hydrophobic interactions in proteins.

5.5 FUNCTIONAL PROPERTIES OF PROTEINS

Food preferences by human beings are based primarily on sensory attributes such as texture, flavor, color, and appearance. The sensory attributes of a food are the net effect of complex interactions among various minor and major components of the food. Proteins generally have a great influence on the sensory attributes of foods. For example, the sensory properties of bakery products are related to the viscoelastic and dough-forming properties of wheat gluten; the textural and succulence characteristics of meat products are largely dependent on muscle proteins (actin, myosin, actomyosin, and several soluble meat proteins); the textural and curd-forming properties of dairy products are due to the unique colloidal structure of casein micelles; and the structure of some cakes and the whipping properties of some desert products depend on the properties of egg-white proteins. The functional roles of various proteins in different food products are listed in Table 5.11 [64]. Functionality of food

TABLE 5.11
Functional Roles of Food Proteins in Food Systems

Function	Mechanism	Food	Protein Type
Solubility	Hydrophilicity	Beverages	Whey proteins
Viscosity	Water binding, hydrodynamic size and shape	Soups, gravies, and salad dressings, deserts	Gelatin
Water binding	Hydrogen bonding, ionic hydration	Meat sausages, cakes, and breads.	Muscle proteins, egg proteins
Gelation	Water entrapment and immobilization, network formation	Meats, gels, cakes, bakeries, cheese	Muscle proteins, egg and milk proteins
Cohesion–adhesion	Hydrophobic, ionic, and hydrogen bonding	Meats, sausages, pasta, baked goods	Muscle proteins, egg proteins, whey proteins
Elasticity	Hydrophobic bonding, disulfide crosslinks	Meats, bakery	Muscle proteins, cereal proteins
Emulsification	Adsorption and film formation at interfaces	Sausages, bologna, soup, cakes, dressings	Muscle proteins, egg proteins, milk proteins
Foaming	Interfacial adsorption and film formation	Whipped toppings, ice cream, cakes, desserts	Egg proteins, milk proteins
Fat and flavor binding	Hydrophobic bonding, entrapment	Low-fat bakery products, doughnuts	Milk proteins, egg proteins, cereal proteins

Source: Kinsella, J. E. et al. 1985. In *New Protein Foods: Seed Storage Protein*s (Altshul, A. M. and H. L. Wilcke, Eds.), Academic Press, London, pp. 107–179.

proteins refers to the physical and chemical properties that influence the performance of proteins in food systems during processing, storage, preparation, and consumption.

The sensory attributes of foods are achieved by complex interactions among various functional ingredients. For instance, the sensory attributes of a cake emanate from gelling/heat-setting, foaming, and emulsifying properties of the ingredients used. Therefore, for a protein to be useful as an ingredient in cakes and other such products, it must possess multiple functionalities. Proteins of animal origin, for example, milk (caseins), egg, and meat proteins, are widely used in fabricated foods. These proteins are mixtures of several proteins with wide ranging physicochemical properties and they are capable of performing multiple functions. For example, egg white possesses multiple functionalities such as gelation, emulsification, foaming, water binding, and heat coagulation, which make it a highly desirable protein in many foods. The multiple functionalities of egg white arise from complex interactions among its protein constituents, namely, ovalbumin, conalbumin, lysozyme, ovomucin, and other albumin-type proteins. Plant proteins (e.g., soy and other legume and oilseed proteins) and other proteins, such as whey proteins, are used to a limited extent in conventional foods. Even though these proteins are also mixtures of several proteins, they do not perform as well as animal proteins in most food products. The exact molecular properties of proteins that are responsible for the various desirable functionalities in food are poorly understood.

The physical and chemical properties that govern protein functionality include size; shape; amino acid composition and sequence; net charge and distribution of charges; hydrophobicity/hydrophilicity ratio; secondary, tertiary, and quaternary structures; molecular flexibility/rigidity; and ability to interact/react with other components. Since proteins possess a multitude of physical and chemical properties, it is difficult to delineate the role of each of these properties with respect to a given functional property.

TABLE 5.12
The Linkage Between the Physicochemical Aspects of Proteins and Their Impact on Functionalities in Foods

General Property	Functions Affected
1. Hydration	Solubility, dispersibility, wettability, swelling, thickening, water absorption, water-holding capacity
2. Surface activity	Emulsification, foaming, flavor binding, pigment binding
3. Hydrodynamic/Rheological	Elasticity, viscosity, cohesiveness, chewiness, adhesion, stickiness, gelation, dough formation, texturization

On an empirical level, the various functional properties of proteins can be viewed as manifestations of three molecular aspects of proteins: (1) hydration properties; (2) protein surface-related properties; and (3) hydrodynamic/rheological properties (Table 5.12). Although much is known about the physicochemical properties of several food proteins, prediction of functional properties from their molecular properties has not been successful. A few empirical correlations between molecular properties and certain functional properties in model protein systems have been established. However, behavior in model systems often is not the same as behavior in real food products. This is attributable, in part, to denaturation of proteins during food fabrication. The extent of denaturation depends on pH, temperature, other processing conditions, and product characteristics. In addition, in real foods, proteins interact with other food components, such as lipids, sugars, polysaccharides, salts, and minor components, and this modifies their functional behavior. Despite these inherent difficulties, considerable progress has been made toward understanding the relationship between various physicochemical properties of protein molecules and their functional properties.

5.5.1 Protein Hydration

Water is an essential constituent of foods. The rheological and textural properties of foods depend on the interaction of water with other food constituents, especially with macromolecules, such as proteins and polysaccharides. Water modifies the physicochemical properties of proteins. For example, the plasticizing effect of water on amorphous and semicrystalline food proteins changes their glass transition temperature (see Chapter 2) and T_d. The glass transition temperature refers to the conversion of a brittle amorphous solid (glass) to a flexible rubbery state, whereas the melting temperature refers to transition of a crystalline solid to a disordered structure.

Many functional properties of proteins, such as dispersibility, wettability, swelling, solubility, thickening/viscosity, water-holding capacity, gelation, coagulation, emulsification, and foaming depend on water–protein interactions. In low and intermediate moisture foods, such as bakery and comminuted meat products, the ability of proteins to bind water is critical to the acceptability of these foods. The ability of a protein to exhibit a proper balance of protein–protein and protein–water interactions is critical to their thermal gelation properties.

Water molecules bind to several groups in proteins. These include charged groups (ion–dipole interactions); backbone peptide groups; the amide groups of Asn and Gln; hydroxyl groups of Ser, Thr, and Tyr residues (all dipole–dipole interactions); and nonpolar residues (dipole–induced dipole interaction and hydrophobic hydration).

The water binding capacity of proteins is defined as grams of water bound per gram of protein when a dry protein powder is equilibrated with water vapor at 90–95% relative humidity. The water binding capacities (also sometimes called hydration capacity) of various polar and nonpolar groups of proteins are given in Table 5.13 [70]. Amino acid residues with charged groups bind about 6 moles of water per residue, the uncharged polar residues bind about 2 mol/residue, and the nonpolar groups

TABLE 5.13
Hydration Capacities of Amino Acid Residues[a]

Amino Acid Residue	Hydration (moles H_2O/mole residue)
Polar	
Asn	2
Gln	2
Pro	3
Ser, The	2
Trp	2
Asp (unionized)	2
Glu (unionized)	2
Tyr	3
Arg (unionized)	3
Lys (unionized)	4
Ionic	
Asp^-	6
Glu^-	7
Tyr^-	7
Arg^+	3
His^+	4
Lys^+	4
Nonpolar	
Ala	1
Gly	1
Phe	0
Val, Ile, Leu, Met	1

[a] Represents unfrozen water associated with amino acid residues based on nuclear magnetic resonance studies of polypeptide.

Source: Kuntz, I. D. 1971. *J. Amer. Chem. Soc.* **93**:514–516.

bind about 1 mol/residue. The hydration capacity of a protein therefore is related, in part, to its amino acid composition—the greater the number of charged residues, the greater is the hydration capacity. The hydration capacity of a protein can be calculated from its amino acid composition using the empirical equation [71]:

$$a = f_C + 0.4f_P + 0.2f_N \tag{5.47}$$

where a is g water/g protein and f_C, f_P, and f_N are the fractions of the charged, polar, and nonpolar residues, respectively, in the protein. The experimental hydration capacities of several monomeric globular proteins agree very well with those calculated from the above equation. This, however, is not true for oligomeric proteins. Since oligomeric structures involve partial burial of the protein surface at the subunit–subunit interface, calculated values are usually greater than experimental values. On the other hand, the experimental hydration capacity of casein micelles (~4 g water/g protein) is much larger than that predicted by the above equation. This is because of the enormous amount of void space within the casein micelle structure, which imbibes water through capillary action and physical entrapment.

On a macroscopic level, water binding to proteins occurs in a step-wise process. The high-affinity ionic groups are solvated first at low water activity, followed by polar and nonpolar groups. The sequence of steps involved at increasing water activity is presented in Figure 5.19 ([109]; see

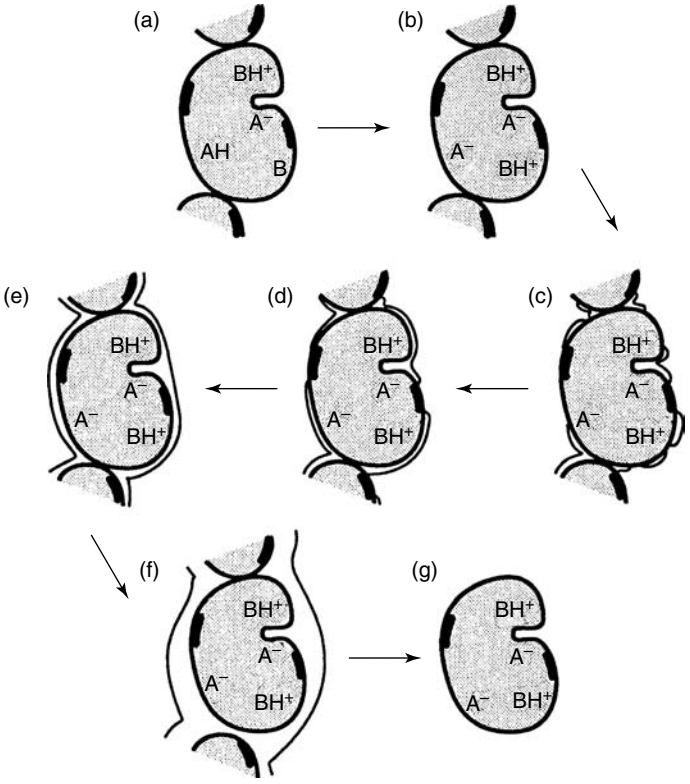


FIGURE 5.19 Sequence of steps involved in hydration of a protein. (a) Unhydrated protein. (b) Initial hydration of charged groups. (c) Water cluster formation near polar and charged sites. (d) Completion of hydration at the polar surfaces. (e) Hydrophobic hydration of nonpolar patches; completion of monolayer coverage. (f) Bridging between protein-associated water and bulk water. (g) Completion of hydrodynamic hydration. (From Rupley, J. A. et al. 1980. In *Water in Polymers* (Rowland, S. P., Ed.), ACS Symp. Ser. 127, American Chemical Society, Washington, D.C., pp. 91–139.)

also Chapter 2). Sorption isotherms of proteins, that is, the amount of water bound per gram of protein as a function of relative humidity is invariably a sigmoidal curve (see Chapter 2). For most proteins, saturated monolayer coverage of water occurs at a water activity (a_w) of about 0.7–0.8, and multilayers of water are formed at $a_w > 0.8$. The saturated monolayer coverage corresponds to about 0.3–0.5 g water/g protein. The saturated monolayer water is primarily associated with ionic, polar, and apolar groups on the surface of the protein. This water is unfreezable, does not take part as a solvent in chemical reactions, and is often referred to as "bound" water, which should be understood to mean water with "hindered" mobility. In the hydration range of 0.07–0.27 g/g, the energy required for desorption of water from the protein surface is only about 0.18 kcal/mol at 25°C. Since the thermal kinetic energy of water at 25°C is about ~1 kcal/mol, which is greater than the free energy of desorption, water molecules in the monolayer are reasonably mobile.

At $a_w = 0.9$, proteins bind about 0.3–0.5 g water/g protein (Table 5.14) [65,71]. At $a_w > 0.9$, liquid (bulk) water condenses into the clefts and crevices of protein molecules, or in the capillaries of insoluble protein systems, such as myofibrils. The properties of this water are similar to those of bulk water. This water is known as hydrodynamic water, which moves with the protein molecule.

Several environmental factors, such as pH, ionic strength, temperature, type of salts, and protein conformation influence the water-binding capacity of proteins. Proteins are least hydrated at their isoelectric pH, where enhanced protein–protein interactions results in minimal interaction with water. Above and below the isoelectric pH, because of the increase in the net charge and repulsive forces,

TABLE 5.14
Hydration Capacities of Various Proteins

Protein	g Water/g Protein
Pure proteins[a]	
Ribonuclease	0.53
Lysozyme	0.34
Myoglobin	0.44
β-Lactoglobulin	0.54
Chymotrypsinogen	0.23
Serum albumin	0.33
Hemoglobin	0.62
Collagen	0.45
Casein	0.40
Ovalbumin	0.30
Commmercial protein preparations[b]	
Whey protein concentrates	0.45–0.52
Sodium caseinate	0.38–0.92
Soy protein	0.33

[a] At 90% relative humidity.
[b] At 95% relative humidity.

Source: From Kinsella, J. E. and P. F. Fox. 1986. *CRC Crit. Rev. Food Sci. Nutr*. **24**:91–139 and Kuntz, I. D. and W. Kauzmann. 1974. *Adv. Protein Chem*. **28**:239–345.

proteins swell and bind more water. The water-binding capacity of most proteins is greater at pH 9–10 than at any other pH. This is due to ionization of sulfhydryl and tyrosine residues. Above pH 10, the loss of positively charged ε-amino groups of lysyl residues results in reduced water binding.

At low concentrations (<0.2 M), salts increase the water binding capacity of proteins. This is because hydrated salt ions, especially the anions, bind (weakly) to charged groups on proteins. At this low concentration, binding of ions to proteins does not affect the hydration shell of the charged groups on the protein, and the increase in water binding essentially comes from water associated with the bound ions. However, at high salt concentrations much of the existing water is bound by salt ions, resulting in dehydration of the protein.

The water binding capacity of proteins generally decreases as the temperature is raised, because of decreased hydrogen bonding and decreased hydration of ionic groups. The water binding capacity of a denatured protein is generally about 10% greater than that of the native protein. This is due to an increase in surface area to mass ratio with exposure of some previously buried hydrophobic groups. If denaturation leads to aggregation of the protein, then its water-binding capacity may actually decrease because of displacement of water by enhanced protein–protein interactions. Denatured food proteins generally exhibit low solubility in water. Their water binding capacities, however, are not drastically different from those in the native state. Thus, water-binding capacity cannot be used to predict the solubility characteristics of proteins. The solubility of a protein is dependent not only on water-binding capacity but also on other thermodynamic factors.

In food applications, the water-holding capacity of a protein is more important than the water binding capacity. Water-holding capacity refers to the ability of the protein to imbibe water and retain it against gravitational force within a protein matrix, such as protein gels or beef and fish muscle. This water refers to the sum of the bound water, hydrodynamic water, and the physically entrapped water. The physically entrapped water contributes more to water-holding capacity than do the bound and hydrodynamic water. However, studies have shown that the water-holding capacity of

proteins is positively correlated with water binding capacity. The ability of proteins to entrap water is associated with juiciness and tenderness of comminuted meat products and desirable textural properties of bakery and other gel-type products.

5.5.2 Solubility

The functional properties of proteins are often affected by protein solubility and those most affected are thickening, foaming, emulsifying, and gelling. Insoluble proteins have very limited uses in food.

The solubility of a protein is the thermodynamic manifestation of the equilibrium between protein–protein and protein–solvent interactions:

$$\text{Protein–Protein} + \text{Water} \rightleftarrows \text{Protein–Water} \tag{5.48}$$

The major interactions that influence the solubility characteristics of proteins are hydrophobic and ionic in nature. Hydrophobic interactions promote protein–protein interactions and result in decreased solubility, whereas ionic interactions promote protein–water interactions and result in increased solubility. Ionic residues introduce two kinds of repulsive forces between protein molecules in solution. The first involves electrostatic repulsion between protein molecules owing to a net positive or negative charge at any pH other than the isoelectric pH; the second involves repulsion between hydration shells around ionic groups.

Bigelow [11] proposed that the solubility of a protein is fundamentally related to the average hydrophobicity of the amino acid residues and the charge frequency. The average hydrophobicity is defined as

$$\Delta G = \sum \Delta g_{\text{residue}}/n \tag{5.49}$$

where $\Delta g_{\text{residue}}$ is the hydrophobicity of each amino acid side chain obtained from the free energy change for transfer from octanol to water (see Section 5.2.1.4), and n is the total number of residues in the protein. The charge frequency is defined as

$$\sigma = \frac{(n^+ + n^-)}{n} \tag{5.50}$$

where n^+ and n^- are the total number of positively and negatively charged residues, respectively, and n is the total number of residues. According to Bigelow [11], the smaller the average hydrophobicity and the larger the charge frequency, the greater will be the solubility of the protein. Although this empirical correlation is true for most proteins, it is not an absolute one. Solubility of a protein is dictated by the hydrophilicity and hydrophobicity of the protein surface that contacts with the surrounding water, rather than the average hydrophobicity and charge frequency of the molecule as a whole. Since a majority of hydrophobic residues are buried in the interior of the protein, only those nonpolar groups that are on the surface would affect the solubility. The fewer the number of surface hydrophobic patches, the greater the solubility.

Based on solubility characteristics, proteins are classified into four categories. *Albumins* are those that are soluble in water at pH 6.6 (e.g., serum albumin, ovalbumin, and α-lactalbumin), *globulins* are those that are soluble in dilute salt solutions at pH 7.0 (e.g., glycinin, phaseolin, and β-lactoglobulin), *glutelins* are those that are soluble only in acid (pH 2) and alkaline (pH 12) solutions (e.g., wheat glutelins), and *prolamines* are those soluble in 70% ethanol (e.g., zein and gliadins). Both prolamines and glutelins are highly hydrophobic proteins.

In addition to these intrinsic physicochemical properties, solubility is influenced by several solution conditions, such as pH, ionic strength, temperature, and the presence of organic solvents.

5.5.2.1 pH and Solubility

At pH values below and above the isoelectric pH, proteins carry a net positive or a net negative charge, respectively. Electrostatic repulsion and hydration of charged residues promote solubilization of the protein. When solubility is plotted against pH, most food proteins exhibit a U-shaped curve. Minimum solubility occurs at about the isoelectric pH of proteins. A majority of food proteins are acidic proteins; that is, the sum of Asp and Glu residues is greater than the sum of Lys, Arg, and His residues. Therefore, they exhibit minimum solubility at pH 4–5 (isoelectric pH), and maximum solubility at alkaline pH. The occurrence of minimum solubility near the isoelectric pH is primarily due to the lack of electrostatic repulsion, which promotes aggregation and precipitation via hydrophobic interactions. Some food proteins are highly soluble at their isoelectric pH, for example, β-lactoglobulin (pI 5.2) and bovine serum albumin (pI 5.3). This is because these proteins contain a large ratio of surface hydrophilic residues to surface nonpolar groups. It should be remembered that even though a protein is electrically neutral at its pI, it still has equal number of positive and negative charges on the surface, contributing to hydrophility of the protein. If the hydrophilicity and the hydration repulsion forces arising from these charged residues are greater than the protein–protein hydrophobic interactions, then the protein will still be soluble at the pI.

Since most proteins are highly soluble at alkaline pH 8–9, protein extraction from plant sources, such as soybean flour, is carried out at this pH. Shown in Figure 5.20 is a typical industrial process for the isolation of soy protein based on its pH-solubility behavior.

Heat denaturation changes the pH-solubility profile of proteins (Figure 5.21). Native whey protein isolate (WPI) is completely soluble in the pH range 2–9, but when heated at 70°C for 1–10 min

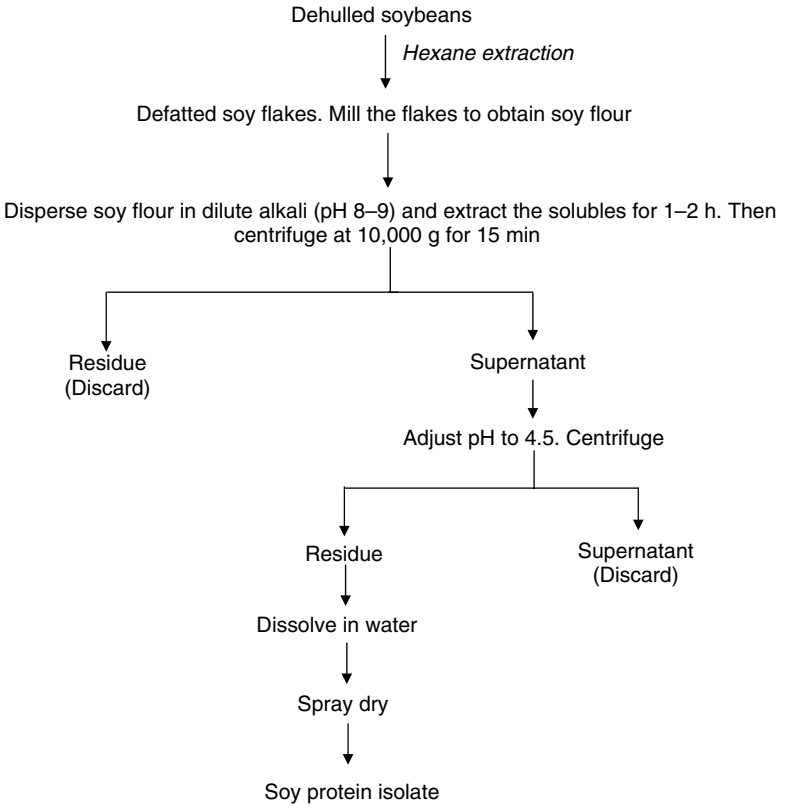


FIGURE 5.20 A typical industrial process for isolation of soy protein from defatted soy flour.

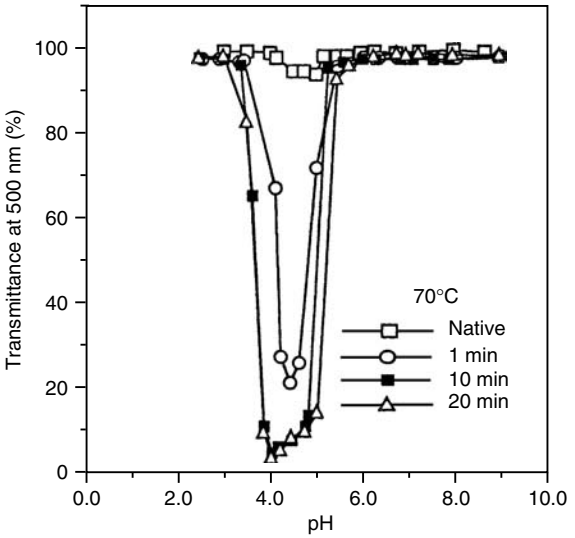


FIGURE 5.21 pH-solubility profile of whey protein isolate solutions heated at 70°C for various times. (From Zhu, H. and S. Damodaran. 1994. *J. Agric. Food Chem.* **42**:846–855.)

a typical U-shaped solubility profile develops with a solubility minimum at pH 4.5. The change in the solubility profile upon heat denaturation is due to an increase in the hydrophobicity of the protein surface as a consequence of unfolding. Unfolding alters the balance between protein–protein and protein–solvent interactions in favor of the former.

5.5.2.2 Ionic Strength and Solubility

The ionic strength of a salt solution is given by:

$$\mu = 0.5 \sum C_i Z_i^2 \tag{5.51}$$

where C_i is concentration of an ion and Z_i is its valence. At low ionic strength (<0.5), ions neutralize charges at the surface of proteins. This charge screening affects solubility in two different ways, depending on the characteristics of the protein surface. Solubility decreases for those proteins that contain a high incidence of nonpolar patches, and it increases for those that do not. The former behavior is typical for soy proteins and the latter behavior is exhibited by β-lactoglobulin. While the decrease in solubility is caused by enhanced hydrophobic interactions, the increase in solubility is caused by a decrease in the ionic activity of the protein macroion. At ionic strength >1.0, salts have ion specific effects on protein solubility. As salt concentration is increased, sulfate and fluoride salts progressively decrease solubility (salting out), whereas bromide, iodide, thiocyanate, and perchlorate salts increase solubility (salting in). At constant ionic strength, relative effectiveness of various ions on solubility follows the Hofmeister series with anions promoting solubility in the order $SO_4^= < F^- < Cl^- < Br^- < I^- < ClO_4^- < SCN^-$ and cations decreasing solubility in the order $NH_4^+ < K^+ < Na^+ < Li^+ < Mg^{2+} < Ca^{2+}$. This behavior is analogous to the effects of salts on the thermal denaturation temperature of proteins (see Section 5.4).

Generally, solubility of proteins in salt solutions follows the relation:

$$\log\left(\frac{S}{S_0}\right) = \beta - K_S C_S, \tag{5.52}$$

where S and S_0 are solubilities of the protein in the salt solution and in water, respectively, K_S is the salting out constant, C_S is molar concentration of salt, and β is a constant characteristic of only protein. K_S is positive for salting-out-type of salts and negative for salting-in-type of salts.

5.5.2.3 Temperature and Solubility

At constant pH and ionic strength, the solubility of most proteins generally increases with temperature between 0°C and 40°C. Exceptions occur with highly hydrophobic proteins, such as β-casein and some cereal proteins, which exhibit a negative relationship with temperature. Above 40°C, the increase in thermal kinetic energy causes protein unfolding (denaturation), exposure of nonpolar groups, aggregation and precipitation, that is, decreased solubility.

5.5.2.4 Organic Solvents and Solubility

Addition of organic solvents, such as ethanol or acetone, lowers the permittivity of an aqueous medium. This increases intra- and intermolecular electrostatic forces, both repulsive as well as attractive. The repulsive intramolecular electrostatic interactions cause unfolding of the protein molecule. In the unfolded state, the low permittivity of the medium promotes intermolecular hydrogen bonding between the exposed peptide groups and attractive intermolecular electrostatic interactions between oppositely charged groups. These intermolecular polar interactions lead to precipitation of the protein in organic solvents or reduced solubility in an aqueous medium. The role of hydrophobic interactions in causing precipitation in organic solvents is minimal because of the solubilizing effect of organic solvents on nonpolar residues. One exception is prolamine-type proteins. These proteins are so hydrophobic that they are soluble only in 70% ethanol.

Since solubility of proteins is intimately related to their structural states, it is often used as a measure of the extent of denaturation during extraction, isolation, and purification processes. It is also used as an index of the potential applications of proteins. Commercially prepared protein concentrates and isolates show a wide range of solubility. The solubility characteristics of these protein preparations are expressed as *protein solubility index* (PSI) or *protein dispersibility index* (PDI). Both of these terms express the percentage (%) of soluble protein present in a protein sample. The PSI of commercial protein isolates varies from 25% to 80%.

5.5.3 Interfacial Properties of Proteins

Several natural and processed foods are either foam or emulsion-type products. These types of dispersed systems are unstable unless a suitable amphiphilic substance is present at the interface between the two phases (see Chapter 13). Proteins are amphiphilic molecules and they migrate spontaneously to an air/water interface or an oil–water interface. This spontaneous migration of proteins from a bulk liquid to an interface indicates that the free energy of proteins is lower at the interface than it is in the bulk aqueous phase. Thus, when equilibrium is established, the concentration of protein in the interfacial region is always much greater than it is in the bulk aqueous phase. Unlike small molecular-weight surfactants, proteins form a highly viscoelastic film at an interface, which has the ability to withstand mechanical shocks during storage and handling. Thus, protein-stabilized foams and emulsions are more stable than those prepared with small molecule surfactants, and because of this, proteins are extensively used for these purposes.

Although all proteins are amphiphilic, they differ significantly in their surface-active properties. The differences in the surface-active properties among proteins cannot be attributed to differences in the ratio of hydrophobic to hydrophilic residues. If a large hydrophobicity/hydrophilicity ratio were the primary determinant of the surface activity of proteins, then plant proteins that contain more than 40% hydrophobic amino acid residues should be better surfactants than albumin-type proteins, such as ovalbumin and bovine serum albumin, which contain $<30\%$ hydrophobic amino

acid residues. On the contrary, ovalbumin and serum albumin are better emulsifying and foaming agents than are soy proteins and other plant proteins. Furthermore, average hydrophobicity of most proteins fall within a narrow range, yet they exhibit remarkable differences in their surface activity. It must be concluded, therefore, that differences in surface activity are related primarily to differences in protein conformation. The conformational factors of importance include stability/flexibility of the polypeptide chain, ease of adaptability to changes in the environment, and distribution pattern of hydrophilic and hydrophobic groups on the protein surface. All these conformational factors are interdependent, and they collectively have a large influence on the surface activity of proteins.

It has been shown that desirable surface-active proteins have three attributes: (1) ability to rapidly adsorb to an interface; (2) ability to rapidly unfold and reorient at an interface; and (3) an ability, once at the interface, to interact with the neighboring molecules and form a strong cohesive, viscoelastic film that can withstand thermal and mechanical motions [31].

Formation and stabilization of foams and emulsions requires the presence of a surfactant that can effectively reduce the interfacial tension between the air/oil and aqueous phases. This can be achieved by using either small surfactants, such as lecithin, monoacylglycerol, and so forth, or macromolecules, such as proteins. At equivalent concentration at an interface, proteins are generally less effective than small surfactants in decreasing the interfacial tension. Typically, most proteins decrease the tension at air–water and oil–water interfaces by about 15 mN m^{-1} at saturated monolayer coverage, compared to 30–40 mN m^{-1} for small molecule surfactants. The inability of proteins to greatly reduce the interfacial tension is related to their complex structural properties. Though proteins contain hydrophilic and hydrophobic groups in their primary structure, there are no clearly defined hydrophilic head and hydrophobic tail as found in lecithin or monoacylglycerol. These groups are randomly spread all over the primary structure of proteins, and in the tertiary folded conformation some of the hydrophobic residues exist as segregated patches on the protein surface while a majority of them are in fact buried in the interior of the protein.

The pattern of distribution of hydrophilic and hydrophobic patches on a protein surface affects its rapidity of adsorption to the air–water or oil–water interface. If the protein surface is extremely hydrophilic and contains no discernable hydrophobic patches, anchoring of the protein at the interface probably will not take place because the protein surface will have a lower free energy in the aqueous phase than at the interface. As the number of hydrophobic patches on the protein surface is increased, spontaneous adsorption to an interface becomes more probable (Figure 5.22) [26]. Single hydrophobic residues randomly distributed on the protein surface do not constitute a hydrophobic patch, nor do they possess sufficient interaction energy to strongly anchor the protein at an interface. Even though more than 40% of a typical globular protein's overall accessible surface is covered with nonpolar residues, they will not enhance protein adsorption unless they exist as segregated regions or patches. In other words, the molecular characteristics of the protein surface have an enormous influence on whether a protein will spontaneously adsorb to an interface and how effective it will be as a stabilizer of dispersions.

The mode of adsorption of proteins at an interface is different from that of small molecular-weight surfactants. In the case of small molecule surfactants, such as phospholipids and monoacylglycerols, conformational constraints for adsorption and orientation do not exist because hydrophilic and hydrophobic moieties are present at the opposite ends of the molecule. In the case of proteins, however, the distribution pattern of hydrophobic and hydrophilic patches on the surface and the structural rigidity of the molecule cause constraints to adsorption and orientation. Because of the bulky, folded nature of proteins, once adsorbed, a large portion of the molecule remains in the bulk phase and only a small portion is anchored at the interface (Figure 5.23). The tenacity with which this small portion of the protein molecule remains attached at the interface depends on the number of peptide segments anchored to the interface and the energetics of interaction between these segments and the interface. The protein will be retained at the interface only when the sum of negative free energy changes of segment interactions is much greater than the thermal kinetic energy of the protein molecule. The number of peptide segments anchored at the interface depends, in part, on the conformational

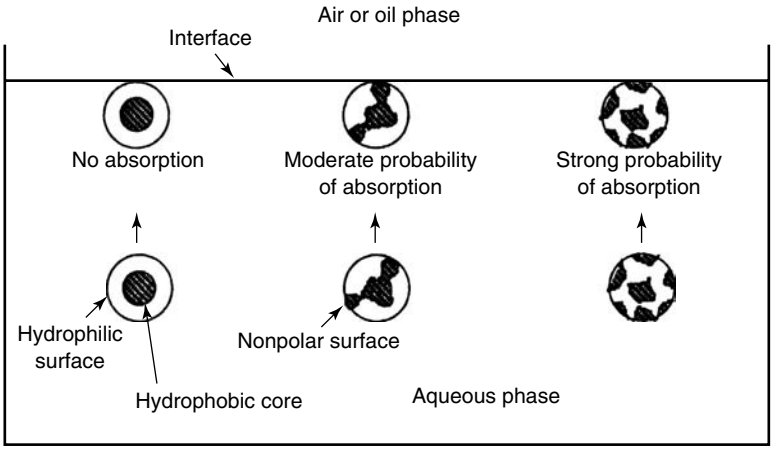


FIGURE 5.22 Schematic representation of the role of surface hydrophobic patches on the probability of adsorption of proteins at the air–water interface. (From Damodaran, S. 1990. *Adv. Food Nutr. Res*. **34**:1–79.)

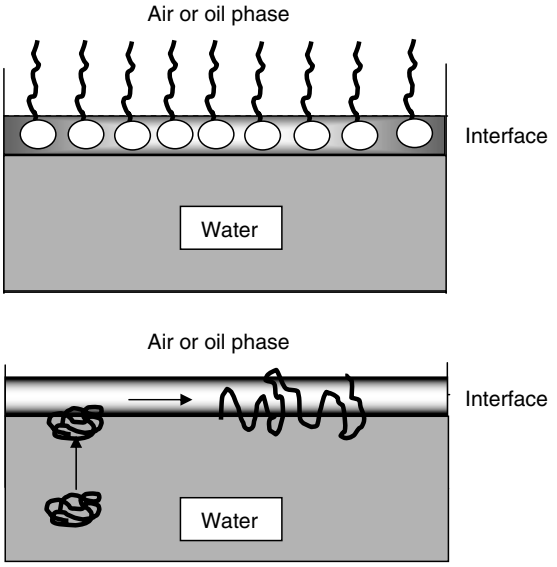


FIGURE 5.23 Difference in the mode of adsorption of a small molecule surfactant and a protein at the air–water or oil–water interface.

flexibility of the molecule. Highly flexible molecules, such as caseins, can undergo rapid conformational changes once they are adsorbed at the interface, enabling additional polypeptide segments to bind to the interface. On the other hand, rigid globular proteins such as lysozyme and soy protein cannot undergo extensive conformational changes at the interface.

At interfaces, polypeptide chains assume three distinct configurations: trains, loops, and tails (Figure 5.24) [26]. The trains are segments that are in direct contact with the interface, loops are segments of the polypeptide that are suspended in the aqueous phase, and tails are N- and C-terminal segments of the protein that are usually located in the aqueous phase. The relative distribution of these three configurations depends on the conformational characteristics of the protein. The greater the proportion of polypeptide segments in a train configuration, the stronger is the binding, and the lower is the interfacial tension.

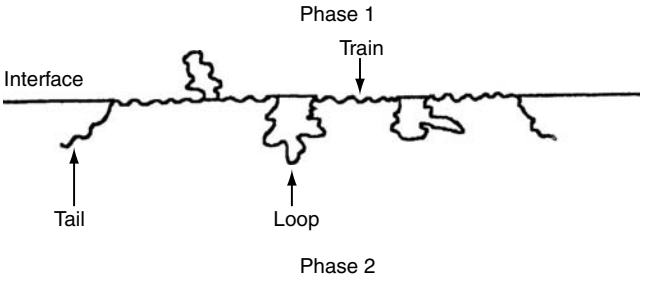


FIGURE 5.24 The various configurations of a flexible polypeptide at an interface. (From Damodaran, S. 1990. *Adv. Food Nutr. Res*. **34**:1–79.)

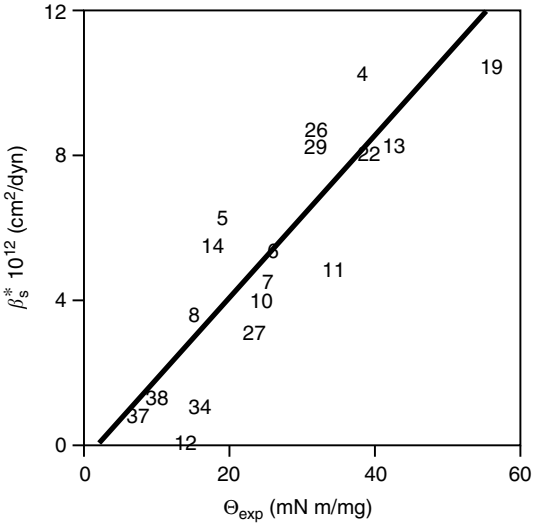


FIGURE 5.25 Relationship between adiabatic compressibility and surface activity of proteins. The numbers in the plot refer to the identities of proteins (see Reference 106 for further details.)

The single most important molecular property that impacts surface activity of proteins is molecular flexibility. This relates to a protein's innate ability to undergo rapid conformational change when it is transferred from one environment to another, for example, from a bulk aqueous phase to an interface. Adiabatic compressibility of proteins is often used as a measure of their molecular flexibility. Investigations on several unrelated proteins have shown that the dynamic surface activity of proteins, that is, the reduction in surface tension caused by one milligram of protein per cm^2 during adsorption from the bulk phase to the air–water interface, is positively and linearly correlated to the adiabatic compressibility (viz., flexibility) of proteins (Figure 5.25) [106]. Rapid conformation change at an interface is essential for the protein to reorient its hydrophobic and hydrophilic residues toward the oil and the aqueous phases and also to maximize the exposure and partitioning of these residues toward the two phases. This will ensure a rapid reduction in the interfacial tension, especially during the initial stages of formation of an emulsion.

The mechanical strength of a protein film at an interface depends on cohesive intermolecular interactions. These include attractive electrostatic interactions, hydrogen bonding, and hydrophobic interactions. Interfacial polymerization of adsorbed proteins via disulfide–sulfhydryl interchange reactions also increases their viscoelastic properties. The concentration of protein in the interfacial film is about 20–25% (w/v), and the protein exists in almost a gel-like state. The balance of various noncovalent interactions is crucial to the stability and viscoelastic properties of this gel-like film.

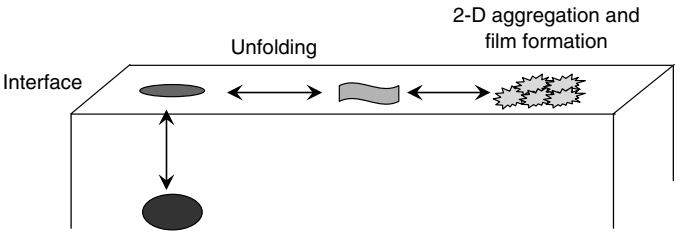


FIGURE 5.26 Schematic illustration of various molecular processes occurring in protein films at interfaces.

For example, if hydrophobic interactions are too strong, this can lead to interfacial aggregation, coagulation, and eventual precipitation of the protein, which will be detrimental to film integrity. If repulsive electrostatic forces are much stronger than attractive interactions, this may prevent formation of a thick, cohesive film. Therefore, a proper balance of attractive, repulsive, and hydration interactions is required to form a stable viscoelastic film. The various molecular processes that occur during adsorption and formation of protein films at interfaces are summarized in Figure 5.26.

The basic principles involved in formation and stability of emulsions and foams are very similar. However, since the energetics of these interfaces is different, the molecular requirements for protein functionality at these interfaces are not the same. In other words, a protein that is a good emulsifier may not be a good foaming agent.

It should now be clear that the behavior of proteins at interfaces is very complex and not well understood. Therefore, the following discussion of the emulsifying and foaming properties of food proteins will be largely qualitative in nature.

5.5.3.1 Emulsifying Properties

The physical chemistry of emulsion formation and the factors affecting creaming, flocculation, coalescence, and stability were reviewed in Chapter 13.

Several natural and processed foods, such as milk, egg yolk, coconut milk, soy milk, butter, margarine, mayonnaise, spreads, salad dressings, frozen desserts, frankfurter, sausage, and cakes are emulsion-type products, where proteins play an important role as an emulsifier. In natural milk, a membrane composed of lipoproteins stabilizes the fat globules. When milk is homogenized, a protein film comprised of casein micelles and whey proteins replaces the lipoprotein membrane. Homogenized milk is more stable against creaming than natural milk is because the casein micelle-whey protein film is stronger than the natural lipoprotein membrane.

5.5.3.1.1 Methods for Determining the Emulsifying Properties of Proteins

The emulsifying properties of food proteins are evaluated by several methods, such as size distribution of oil droplets formed, emulsifying activity, emulsion capacity (EC), and emulsion stability.

Emulsifying activity index: The physical and sensory properties of a protein-stabilized emulsion depend on the size of the droplets formed and the total interfacial area created. The average droplet size of emulsions can be determined by several methods, such as light microscopy (not very reliable), electron microscopy, light scattering (photon correlation spectroscopy), or use of a Coulter counter. Knowing mean droplet size, total interfacial area can be obtained from the relation:

$$A = \frac{3\phi}{R} \tag{5.53}$$

where ϕ is the volume fraction of the dispersed phase (oil) and R is the mean radius of the emulsion particles. If m is the mass of the protein, then the Emulsifying Activity Index (EAI), that is, the

interfacial area created per unit mass of protein is

$$\text{EAI} = \frac{3\phi}{Rm}. \tag{5.54}$$

Another simple and more practical method to determine EAI of proteins is the turbidimetric method [99]. The turbidity of an emulsion is given by:

$$T = \frac{2.303A}{l} \tag{5.55}$$

where A is absorbance and l is path length. According to Mie theory of light scattering, the interfacial area of an emulsion is twice its turbidity. If ϕ is the volume fraction of the oil, and C is the weight of protein per unit volume of the aqueous phase, then the EAI of the protein is given by:

$$\text{EAI} = \frac{2T}{(1-\phi)C}. \tag{5.56}$$

It should be mentioned that in the original article [99], ϕ, instead of $(1 - \phi)$ was used in the denominator of the above equation. The above expression is the correct one because ϕ is defined as the oil volume fraction, and thus $(1 - \phi)C$ is the total mass of protein in a unit volume of the emulsion [17]. Although this method is simple and practical, the main drawback is that it is based on measurement of turbidity at one single wavelength, 500 nm. Since the turbidity of food emulsions is wavelength dependent, the interfacial area obtained from turbidity at 500 nm is not very accurate. Therefore, use of the above equation to estimate mean particle diameter or the number of emulsion particles present in the emulsion gives results that are not very reliable. However, the method can be used for qualitative comparison of emulsifying activities of different proteins, or changes in the emulsifying activity of a protein after various treatments.

Protein load: The amount of protein adsorbed at the oil–water interface of an emulsion has a bearing on its stability. To determine the amount of protein adsorbed, the emulsion is centrifuged, the aqueous phase is separated, and the cream phase is repeatedly washed and centrifuged to remove any loosely adsorbed proteins. The amount of protein adsorbed to the emulsion particles is determined from the difference between the total protein initially present in the emulsion and the amount present in the wash fluid from the cream phase. Knowing the total interfacial area of the emulsion particles, the amount of protein adsorbed/m^2 of the interfacial area can be calculated. Generally, the protein load is in the range of about 1–3 mg/m^2 of interfacial area. As the volume fraction of the oil phase is increased, the protein load decreases at constant protein content in the total emulsion. For high-fat emulsions and small-sized droplets, more protein is obviously needed to adequately coat the interfacial area and stabilize the emulsion.

Emulsion capacity (EC): EC is the volume (mL) of oil that can be emulsified per gram of protein before phase inversion (a change from oil-in-water emulsion to water-in-oil) occurs. This method involves addition of oil or melted fat at a constant rate and temperature to an aqueous protein solution that is continuously agitated in a food blender. Phase inversion is detected by an abrupt change in viscosity or color (usually a dye is added to the oil), or by an increase in electrical resistance. For a protein-stabilized emulsion, phase inversion usually occurs when ϕ is about 0.65–0.85. Inversion is not instantaneous, but is preceded by formation of a water-in-oil-in-water double emulsion. Since EC is expressed as volume of oil emulsified per gram protein at phase inversion, it decreases with increasing protein concentration once a point is reached where unadsorbed protein accumulates in the aqueous phase. Therefore, to compare emulsion capacities of different proteins, EC vs. protein concentration profiles should be used instead of EC at a specific protein concentration.

Emulsion stability: Protein stabilized emulsions are often stable for days. Thus, a detectable amount of creaming or phase separation is usually not observed in a reasonable amount of time when samples are stored at atmospheric conditions. Therefore, drastic conditions, such as storage at elevated temperature or separation under centrifugal force is often used to evaluate emulsion stability. If centrifugation is used, stability is then expressed as percent decrease in interfacial area (i.e., turbidity) of the emulsion, or percent volume of cream separated, or as the fat content of the cream layer. More often, however, emulsion stability is expressed as

$$\text{ES} = \frac{\text{volume of cream layer}}{\text{total volume of emulsion}} \times 100 \tag{5.57}$$

where the volume of the cream layer is measured after a standardized centrifugation treatment. A common centrifugation technique involves centrifugation of a known volume of emulsion in a graduated centrifuge tube at 1300 g for 5 min. The volume of the separated cream phase is then measured and expressed as percentage of the total volume. Sometimes centrifugation at a relatively low gravitational force (180 g) for a longer time (15 min) is used to avoid coalescence of droplets.

The turbidimetric method (see above) can also be used to evaluate emulsion stability. In this case stability is expressed as Emulsion Stability Index (ESI), which is defined as the time to achieve a turbidity of the emulsion that is one-half of the original value.

The methods used to determine emulsion stability are very empirical. The most fundamental quantity related to stability is the change in interfacial area with time, but this is difficult to measure directly.

5.5.3.1.2 Factors Influencing Emulsification

The properties of protein-stabilized emulsions are affected by several factors. These include intrinsic factors, such as pH, ionic strength, temperature, presence of low molecular-weight surfactants, sugars, oil phase volume, type of protein, and the melting point of the oil used; and extrinsic factors such as type of equipment, rate of energy input, and rate of shear. Standardized methods for systematically evaluating the emulsifying properties of proteins have not emerged. Therefore, results among laboratories cannot be accurately compared and this has hampered the understanding of the molecular factors that affect emulsifying properties of proteins.

The general forces involved in the formation and stabilization of emulsion were discussed in Chapter 13. Therefore, only the molecular factors that affect protein-stabilized emulsions need be discussed here.

Solubility plays a role in emulsifying properties, but 100% solubility is not an absolute requirement. While highly insoluble proteins do not perform well as emulsifiers, no reliable relationship exists between solubility and emulsifying properties in the 25–80% solubility range [76]. However, since the stability of a protein film at the oil–water interface is dependent on favorable interactions with both the oil and aqueous phases, some degree of solubility is likely to be necessary. The minimum solubility requirement for good performance may vary among proteins. In meat emulsions, such as in sausage and frankfurter, solubilization of myofibrillar proteins in 0.5 M NaCl enhances their emulsifying properties. Some commercial soy protein isolates that are isolated by thermal processing have poor emulsifying properties because of their very low solubility.

The formation and stability of protein-stabilized emulsions are affected by pH. Several mechanisms are involved. Generally, proteins that have high solubility at the isoelectric pH (e.g., serum albumin, gelatin, and egg-white proteins) show maximum emulsifying activity and EC at that pH. The lack of net charge and electrostatic repulsive interactions at the isoelectric pH helps maximize protein load at the interface and promotes formation of a highly viscoelastic film, both of which contribute to emulsion stability. However, the lack of electrostatic repulsive interactions among emulsion particles can, in some instances promote flocculation, coalescence, and thus decrease emulsion stability. On the other hand, if the protein is highly hydrated at the isoelectric pH (unusual), then

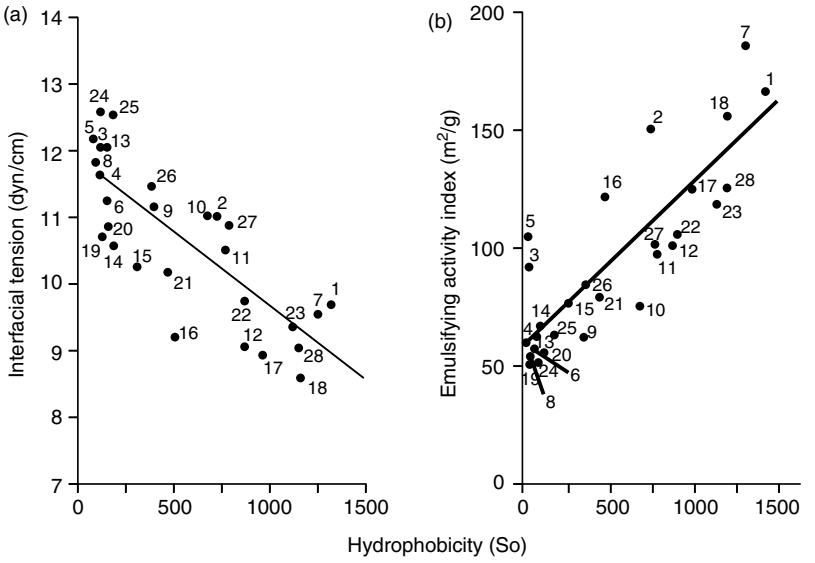


FIGURE 5.27 Correlations of surface hydrophobicity of various proteins with (a) oil–water interfacial tension and (b) EAI. Surface hydrophobicity was determined from the amount of hydrophobic fluorescent probe bound per unit weight of protein. The numbers in the plots represent (1) bovine serum albumin; (2) β-lactoglobulin; (3) trypsin; (4) ovalbumin; (5) conalbumin; (6) lysozyme; (7) κ-casein; (8–12) ovalbumin denatured by heating at 85°C for 1, 2, 3, 4, or 5 min, respectively; (13–18) lysozyme denatured by heating at 85°C for 1, 2, 3, 4, 5, or 6 min, respectively; (19–23) ovalbumin bound to 0.2, 0.3, 1.7, 5.7, or 7.9 mole dodecyl sulfate per mol protein, respectively; (24–28) ovalbumin bound to 0.3, 0.9, 3.1, 4.8, or 8.2 mol linoleate per mole protein, respectively. (From Kato, A. and S. Nakai. 1980. *Biochim. Biophys. Acta* **624**:13–20.)

hydration repulsion forces between emulsion particles may prevent flocculation and coalescence, and thus stabilize the emulsion. Because most food proteins (caseins, commercial whey proteins, meat proteins, and soy proteins) at their isoelectric pH are sparingly soluble, poorly hydrated and lack electrostatic repulsive forces, they are generally poor emulsifiers at this pH. These proteins may, however, be effective emulsifiers when moved away from their isoelectric pH.

The emulsifying properties of proteins show a weak positive correlation with surface hydrophobicity, but not with mean residue hydrophobicity (i.e., kcal mol^{-1} $residue^{-1}$). The ability of various proteins to decrease interfacial tension at the oil–water interface and to increase the EAI is related to their surface hydrophobicity values (Figure 5.27). However, this relationship is by no means perfect. The emulsifying properties of several proteins, such as β-lactoglobulin, α-lactalbumin, and soy proteins, do not show a strong correlation with surface hydrophobicity.

The surface hydrophobicity of proteins is usually determined by measuring the amount of a hydrophobic fluorescent probe, such as *cis*-parinaric acid, that can bind to the protein [62]. Although this method provides some information on the hydrophobicity of the protein surface, it is questionable whether the measured value truly reflects the "hydrophobicity" of the protein surface. The true definition of surface hydrophobicity is that portion of the nonpolar surface of the protein that makes contact with the surrounding bulk water. However, *cis*-parinaric acid is capable of binding only to hydrophobic cavities. These protein cavities are accessible to nonpolar ligands, but they are not accessible to water and may not be accessible to either phase in an oil–water emulsion, unless the protein is able to undergo rapid conformational rearrangement at the interface. The poor correlation of surface hydrophobicity (as measured by *cis*-parinaric acid binding) with the emulsifying properties of some proteins may be related to the fact that *cis*-parinaric acid provides no indication of molecular flexibility. Molecular flexibility at the oil–water interface may be the most important determinant of the emulsifying properties of proteins.

Partial denaturation of proteins prior to emulsification, which does not result in insolublization, usually improves their emulsifying properties. This is due to increased molecular flexibility and surface hydrophobicity. In the unfolded state, proteins containing free sulfhydryl groups and disulfide bonds undergo slow polymerization via disulfide–sulfhydryl interchange reaction [32]. This leads to formation of a highly viscoelastic film at the oil–water interface. Excessive heat denaturation may impair the emulsifying properties by rendering the protein insoluble.

Small molecule emulsifiers, such as phospholipids, which are generally found in foods, compete with proteins for adsorption at the oil–water interface [24,38,67]. Since small molecule surfactants can diffuse rapidly to the interface and lack conformational constraints for reorientation at the interface, they can effectively inhibit adsorption of proteins at high concentrations. If small molecule emulsifiers are added to a protein-stabilized emulsion, they can displace the protein from the interface and cause instability in the emulsion.

Another factor that affects protein-stabilized emulsions is the protein composition. Food proteins in general are mixtures of several protein components. For instance, egg protein is a mixture of five major proteins and several minor protein components. Likewise, whey protein is a mixture of α-lactalbumin, β-lactoglobulin, and several other minor proteins. Seed storage proteins, such as soy protein isolate, contain at least two major protein fractions, viz., legumins and vicillins. During emulsification, the protein components of the mixture compete with each other for adsorption to the interface. The composition of the protein film formed at the interface is dependent on relative surface activities of various protein components of the mixture. For instance, when a 1:1 mixture of α- and β-caseins are allowed to adsorb to the oil–water interface, the amount of α-casein in the protein film at equilibrium is almost twice that of β-casein [30]. At the air–water interface, however, an exactly opposite behavior is observed [6]. Variations in the protein composition of the bulk phase would affect protein composition of the adsorbed film and possibly the stability of the emulsion.

At high concentration, protein mixtures generally exhibit incompatibility of mixing in solution [100]. In mixed protein films at the oil–water interface, where the local protein concentration is in the range of 15–30%, it is likely that two-dimensional phase separation of the proteins can occur with storage time. Evidence for this at the air–water [105,114] and oil–water [30] interfaces has been reported. If distinct phase separation of proteins occurs in mixed protein films around oil droplets, it is conceivable that the interface of such phase-separated regions might act as source of instability in emulsions. However, a direct correlation between thermodynamic incompatibility of mixing of proteins in mixed protein films at the oil–water interface and the kinetic stability of emulsions made of protein mixtures is yet to be determined.

5.5.3.2 Foaming Properties

Foams consist of an aqueous continuous phase and a gaseous (air) dispersed phase. Many processed foods are foam-type products. These include whipped cream, ice cream, cakes, meringue, bread, souffles, mousses, and marshmallow. The unique textural properties and mouthfeel of these products stem from the dispersed tiny air bubbles. In most of these products, proteins are the main surface active agents that help in the formation and stabilization of the dispersed gas phase.

Generally, bubbling, whipping, or shaking a protein solution creates protein-stabilized foams. The foaming property of a protein refers to its ability to form a thin tenacious film at gas–liquid interfaces so that large quantities of gas bubbles can be incorporated and stabilized. Foaming properties are evaluated by several means. The *foamability* or *foaming capacity* of a protein refers to the amount of interfacial area that can be created by the protein. It can be expressed in several ways, such as *overrun* (or steady state foam volume) or *foaming power* (or foam expansion). Overrun is defined as

$$\text{Overrun} = \frac{\text{Volume of foam}}{\text{Volume of initial liquid}} \times 100 \tag{5.58}$$

TABLE 5.15
Comparative Foaming Power of Protein Solutions

Protein Type	Foaming Power[a] at 0.5% Protein Conc. (w/v) (%)
Bovine serum albumin	280
Whey protein isolate	600
Egg albumen	240
Ovalbumin	40
Bovine plasma	260
β-Lactoglobulin	480
Fibrinogen	360
Soy protein (enzyme hydrolyzed)	500
Gelatin (acid-processed pigskin)	760

[a] Calculated according to Equation 5.56.

Source: From Poole, S. et al. 1984. *J. Sci. Food Agric.* **35**:701–711.

The foaming power (FP), is expressed as

$$\text{FP} = \frac{\text{Volume of gas incorporated}}{\text{Volume of liquid}} \times 100 \tag{5.59}$$

Foaming power generally increases with protein concentration until a maximum value is attained. It is also affected by the method used for foam formation. FP at a given protein concentration is often used as a basis for comparing the foaming properties of various proteins. The FPs of various proteins at pH 8.0 are given in Table 5.15 [82].

Foam stability refers to the ability of protein to stabilize foam against gravitational and mechanical stresses. Foam stability is often expressed as the time required for 50% of the liquid to drain from foam or for a 50% reduction in foam volume. These are very empirical methods, and they do not provide fundamental information about the factors that affect foam stability. The most direct measure of foam stability is the reduction in foam interfacial area as a function of time. This can be done as follows. According to the Laplace principle, the internal pressure of a bubble is greater than the external (atmospheric) pressure, and under stable conditions the pressure difference, ΔP, is

$$\Delta P = p_{\text{i}} - p_{\text{o}} = \frac{4\gamma}{r} \tag{5.60}$$

where p_{i} and p_{o} are the internal and external pressures, respectively, r is radius of the foam bubble, and γ is surface tension. According to the above equation, the pressure inside a closed vessel containing foam will increase when the foam collapses. The net change in the pressure is [92]

$$\Delta P = \frac{-2\gamma \Delta A}{3V} \tag{5.61}$$

where V is the total volume of the system, ΔP is the pressure change, and ΔA is the net change in interfacial area resulting from the fraction of collapsed foam. The initial interfacial area of the foam is given by:

$$A_0 = \frac{3V\Delta P_\infty}{2\gamma} \tag{5.62}$$

where ΔP_∞ is the net pressure change when the entire foam is collapsed. The A_0 value is a measure of foamability, and the rate of decrease of A with time can be used as a measure of foam stability. This approach has been used to study the foaming properties of food proteins [133,135].

The *strength* or *stiffness* of the foam refers to the maximum weight a column of foam can withstand before it collapses. Measuring foam viscosity also assesses this property.

5.5.3.2.1 Environmental Factors Influencing Foam Formation and Stability

pH: Several studies have shown that protein-stabilized foams are more stable at the isoelectric pH of the protein than at any other pH, provided there is no insolublization of the protein at pI. At or near the isoelectric pH region, the lack of repulsive interactions promotes favorable protein–protein interactions and formation of a viscous film at the interface. In addition, an increased amount of protein is adsorbed to the interface at the pI because of lack of repulsion between the interface and the adsorbing molecules. These two factors improve both foamability and foam stability. If the protein is sparingly soluble at pI, as most food proteins are, then only the soluble protein fraction will be involved in foam formation. Since the concentration of this soluble fraction is very low, the amount of foam formed will be less, but the stability will be high. Although the insoluble fraction does not contribute to foamability, adsorption of these insoluble protein particles may stabilize the foam, probably by increasing cohesive forces in the protein film. Generally, adsorption of hydrophobic particles increases the stability of foams. At pH other than pI, foamability of proteins is often good, but foam stability is poor. Egg-white proteins exhibit good foaming properties at pH 8–9 and at their isoelectric pH 4–5.

Salts: The effects of salts on the foaming properties of proteins depend on the type of salt and the solubility characteristics of the protein in that salt solution. The foamability and foam stability of most globular proteins, such as bovine serum albumin, egg albumin, gluten, and soy proteins increase with increasing concentration of NaCl. This behavior is usually attributed to neutralization of charges by salt ions. However, some proteins, such as whey proteins, exhibit the opposite effect: both foamability and foam stability decrease with increasing concentration of NaCl (Table 5.16) [136]. This is attributed to salting-in of whey proteins, especially β-lactoglobulin. Proteins that are salted-out in a given salt solution generally exhibit improved foaming properties; whereas, those that are salted-in generally exhibit poor foaming properties. Divalent cations, such as Ca^{2+} and Mg^{2+}, dramatically improve both foamability and foam stability at 0.02–0.4 M concentration. This is primarily due to cross-linking of protein molecules and creation of films with better viscoelastic properties [134].

TABLE 5.16
Effect of NaCl on Foamability and Foam Stability of Whey Protein Isolate

NaCl Concentration (M)	Total Interfacial Area (cm^2/ml of foam)	Time for 50% Collapse of Initial Area (s)
0.00	333	510
0.02	317	324
0.04	308	288
0.06	307	180
0.08	305	165
0.10	287	120
0.15	281	120

Source: Compiled from Zhu, H. and S. Damodaran. 1994. *J. Food Sci*. **59**:554–560.

Sugars: Addition of sucrose, lactose, and other sugars to protein solutions often impairs foamability, but improves foam stability. The positive effect of sugars on foam stability is due to increased bulk phase viscosity, which reduces the rate of drainage of the lamella fluid. The depression in foam overrun is mainly due to enhanced stability of protein structure in sugar solutions. Because of this, the protein molecule is less able to unfold upon adsorption at the interface. This decreases the ability of the protein to reduce interfacial tension, produce large interfacial areas and large foam volume during whipping. In sugar containing, foam-type dessert products, such as meringues, souffles, and cakes, it is preferable to add sugar after whipping when possible. This will enable the protein to adsorb, unfold, and form a stable film, and then the added sugar will increase foam stability by increasing the viscosity of the lamella fluid.

Lipids: Lipids, especially phospholipids, when present at concentrations >0.5%, markedly impair the foaming properties of proteins. Because lipids are more surface-active than proteins, they readily adsorb at the air–water interface and inhibit adsorption of proteins during foam formation. Since lipid films lack the cohesive and viscoelastic properties necessary to withstand the internal pressure of the foam bubbles, the bubbles rapidly expand, then collapse during whipping. Thus, lipid-free whey protein concentrates (WPC) and isolates, soy proteins, and egg proteins without egg yolk display better foaming properties than do lipid-contaminated preparations.

Protein concentration: Several properties of foams are influenced by protein concentration. The greater the protein concentration, the stiffer is the foam. Foam stiffness results from small bubble size and high viscosity. The stability of the foam is enhanced by greater protein concentrations because this increases viscosity and facilitates formation of a multilayer, cohesive, protein film at the interface. Foamability generally reaches a maximum value at some point during increase of protein concentration. Some proteins, for example, serum albumin, are able to form relatively stable foams at 1% protein concentration, whereas proteins such as WPI and soy proteins require a minimum of 2–5% to form relatively stable foam. Generally, most proteins display maximum foamability at 2–8% concentration. The interfacial concentration of proteins in foams is about 2–3 mg/m^2.

Partial heat denaturation improves the foaming properties of proteins. For instance, heating of WPI at 70°C for 1 min improves, whereas heating at 90°C for 5 min decreases foaming properties even though the heated proteins remain soluble in both instances [135]. The decrease in foaming properties of WPI heated at 90°C is due to extensive polymerization of the protein via disulfide-sulfhydryl interchange reactions. The highly cross-linked and polymerized protein is unable to adsorb to the air–water interface during foaming.

The method of foam generation influences the foaming properties of proteins. Air introduction by bubbling or sparging usually results in "wet" foam with a relatively large bubble size. Whipping at moderate speed generally results in foam with small-sized bubbles because the shearing action results in partial denaturation of the protein before adsorption occurs. However, whipping at high shear rate or "overbeating" can decrease foaming power because of extensive denaturation, aggregation, and precipitation of proteins.

Some foam-type food products, such as marshmallow, cakes, and bread, are heated after the foam is formed. During heating, expansion of air and decreased viscosity can cause bubble rupture and collapse of the foam. In these instances, the integrity of the foam depends on gelation of the protein film at the interface so sufficient mechanical strength is developed to stabilize the foam. Gelatin, gluten, and egg white, which display good foaming and gelling properties, are highly suitable for this purpose.

5.5.3.2.2 Molecular Properties Influencing Foam Formation and Stability

For a protein to perform effectively as a foaming agent or an emulsifier it must meet the following basic requirements: (1) it must be able to rapidly adsorb to the air–water interface, (2) it must readily

unfold and rearrange at the interface, and (3) it should be able to form a viscous cohesive film through intermolecular interactions. The molecular properties that affect foaming properties are molecular flexibility, charge density and distribution, and hydrophobicity.

The free energy of the air–water interface is significantly greater than that of the oil–water interface. Therefore, to stabilize the air–water interface, the protein must have the ability to rapidly adsorb to the freshly created interface, and instantaneously decrease the interfacial tension to a low value. The lowering of interfacial tension is dependent on the ability of the protein to rapidly unfold, rearrange, and expose hydrophobic groups at the interface. Random-coil-type proteins, such as β-casein, perform well in this manner. On the other hand, tightly folded globular proteins, such as lysozyme, adsorb very slowly, only partially unfold, and reduce the surface tension only slightly [130]. Lysozyme is, therefore, a poor foaming agent. Thus, molecular flexibility at the interface is quintessential for good performance as a foaming agent.

Apart from molecular flexibility, hydrophobicity also plays a role in foamability of proteins. The foaming power of proteins is positively correlated with the *mean* hydrophobicity. However, the foaming power of proteins varies curvilinearly with *surface* hydrophobicity, and a significant correlation does not exist between these two properties at hydrophobicity values of greater than 1000 [63]. This indicates that a surface hydrophobicity of at least 1000 is needed for initial adsorption of proteins at the air–water interface, whereas, once adsorbed, the ability of the protein to create more interfacial area during foam formation depends on the mean hydrophobicity of the protein.

A protein that displays good foamability need not be a good foam stabilizer. For example, although β-casein exhibits excellent foamability, the stability of the foam is poor. On the other hand, lysozyme exhibits poor foamability, but its foams are very stable. Generally, proteins that possess good foaming power do not have the ability to stabilize foam, and proteins that produce stable foams often exhibit poor foaming power. It appears that foamability and stability are influenced by two different sets of molecular properties of proteins that are often antagonistic. Whereas foamability is affected by rate of adsorption, flexibility, and hydrophobicity, stability depends on the rheological properties of the protein film. The rheological properties of films depend on hydration, thickness, protein concentration, and favorable intermolecular interactions. Proteins that only partially unfold and retain some degree of folded structure usually form thicker, denser films, and more stable foams (e.g., lysozyme and serum albumin) than do those that completely unfold (e.g., β-casein) at the air–water interface. In the former case, the folded structure extends into the subsurface in the form of loops. Noncovalent interactions, and possibly disulfide cross-linking, between these loops promote formation of a gel network, which has excellent viscoelastic and mechanical properties. For a protein to possess good foamability and foam stability it should have an appropriate balance between flexibility and rigidity, should easily undergo unfolding, and should engage in abundant cohesive interactions at the interface. However, what extent of unfolding is desirable for a given protein is difficult, if not impossible, to predict. In addition to these factors, foam stability usually exhibits an inverse relationship with the charge density of proteins. High charge density apparently interferes with formation of a cohesive film.

Most food proteins are mixtures of various proteins, and therefore their foaming properties are influenced by interaction between the protein components at the interface. The excellent whipping properties of egg white are attributed to interactions between its protein components, such as ovalbumin, conalbumin, and lysozyme. Several studies have indicated that the foaming properties of acidic proteins can be improved by mixing them with basic proteins, such as lysozyme and clupeine [102]. This enhancing effect seems to be related to the formation of an electrostatic complex between the acidic and basic proteins.

Limited enzymatic hydrolysis of proteins generally improves their foaming properties. This is because of increased molecular flexibility and greater exposure of hydrophobic groups. However, extensive hydrolysis impairs foamability because low molecular weight peptides cannot form a cohesive film at the interface.

5.5.4 Flavor Binding

Proteins themselves are odorless. However, they can bind flavor compounds, and thus affect the sensory properties of foods. Several proteins, especially oilseed proteins and WPCs, carry undesirable flavors, which limits their usefulness in food applications. These off-flavors are mainly due to aldehydes, ketones, and alcohols generated by oxidation of unsaturated fatty acids. Upon formation, these carbonyl compounds bind to proteins and impart characteristic off-flavors. For example, the beany and grassy flavor of soy protein preparations is attributed to the presence of hexanal. The binding affinity of some of these carbonyls is so strong that they resist even solvent extraction. A basic understanding of the mechanism of binding of off-flavors to proteins is needed so that appropriate methods can be developed for their removal.

The flavor-binding property of proteins also has desirable aspects, because they can be used as flavor carriers or flavor modifiers in fabricated foods. This is particularly useful in meat analogues containing plant proteins, where successful simulation of a meat-like flavor is essential for consumer acceptance. In order for a protein to function as a good flavor carrier, it should bind flavors tightly, retain them during processing, and release them during mastication of food in the mouth. However, proteins do not bind all flavor compounds with equal affinity. This leads to uneven and disproportionate retention of some flavors and undesirable losses during processing. Because protein-bound flavorants do not contribute to taste and aroma unless they are released readily in the mouth, knowledge of the mechanisms of interaction and binding affinity of various flavorants is essential if effective strategies for producing flavor-protein products or for removing off-flavors from protein isolates are to be devised.

5.5.4.1 Thermodynamics of Protein–Flavor Interactions

In water-flavor model systems, addition of proteins causes a reduction in the headspace concentration of flavor compounds. This is due to binding of flavors to proteins. The mechanism of flavor binding to proteins depends upon the moisture content of the protein sample, but interactions are normally noncovalent. Dry protein powders bind flavors mainly via van der Waals, hydrogen bonding, and electrostatic interactions. Physical entrapment within capillaries and crevices of dry protein powders may also contribute to flavor properties of dry protein powders. In liquid or high moisture foods, the mechanism of flavor binding by proteins primarily involves interaction of the nonpolar flavor compounds (ligands) with hydrophobic patches/cavities on the protein surface. In addition to hydrophobic interactions, flavor compounds with polar head groups, such as hydroxyl and carboxyl groups may also interact with proteins via hydrogen bonding and electrostatic interactions. After binding to the surface hydrophobic regions, aldehydes and ketones may be able to diffuse into the hydrophobic interior of the protein molecule.

Flavor–protein interaction is usually completely reversible. However, aldehydes, can covalently bind to the amino group of lysine side chains and this interaction is nonreversible. However, only the noncovalently bound fraction can contribute to aroma and taste of the protein product.

The extent of flavor binding by hydrated proteins depends on the number of hydrophobic binding regions available on the protein surface [28]. The binding sites are usually made up of groups of hydrophobic residues segregated in the form of a well-defined cavity. Single nonpolar residues on the protein surface are less likely to act as binding sites. Under equilibrium conditions, the reversible noncovalent binding of a flavor compound with proteins follows the Scatchard equation:

$$\frac{\upsilon}{[L]} = nK - \upsilon K \tag{5.63}$$

where υ is moles of ligand bound per mole of protein, n is the total number of binding sites per mole of protein, $[L]$ is the free ligand concentration at equilibrium, and K is the equilibrium binding constant (M^{-1}). According to this equation, a plot of $\upsilon/[L]$ vs. υ will be a straight line; the values

TABLE 5.17
Thermodynamic Constants for Binding of Carbonyl Compounds to Proteins

Protein	Carbonyl Compound	n (moles/mole)	K (M^{-1})	ΔG (kcal/mol)
Serum albumin	2-Nonanone	6	1800	−4.4
	2-Heptanone	6	270	−3.3
β-Lactoglobulin	2-Heptanone	2	150	−3.0
	2-Octanone	2	480	−3.7
	2-Nonanone	2	2440	−4.7
Soy Protein				
Native	2-Heptanone	4	110	−2.8
	2-Octanone	4	310	−3.4
	2-Nonanone	4	930	−4.1
	5-Nonanone	4	541	−3.8
	Nonanal	4	1094	−4.2
Partially denatured	2-Nonanone	4	1240	−4.3
Succinylated	2-Nonanone	2	850	−4.0

n, number of binding sites in native state; K, equilibrium binding constant.

Source: Compiled from Damodaran, S. and J. E. Kinsella. 1980. *J. Agric. Food Chem.* **28**:567–571; Damodaran, S. and J. E. Kinsella. 1981. *J. Agric. Food Chem.* **29**:1249–1253; and O'Neill, T. E. and J. E. Kinsella. 1987. *J. Agric. Food Chem.* **35**:770–774.

of K and n can be obtained from the slope and the intercept, respectively. The free energy change for binding of ligand to protein is obtained from the equation

$$\Delta G = -RT \ln K,$$

where R is the gas constant and T is absolute temperature. The thermodynamic constants for the binding of carbonyl compounds to various proteins are presented in Table 5.17 [28,29,95]. The binding constant increases by about threefold for each methylene group increment in chain length, with a corresponding free energy change of −0.55 kcal/mol per CH_2 group. This indicates that the binding is hydrophobic in nature.

It is assumed in the Scatchard relationship that all ligand-binding sites in a protein have the same affinity, and that no conformational changes occur upon binding of the ligand to these sites. Contrary to the latter assumption, proteins generally do undergo a modest conformational change upon binding of flavor compounds. Diffusion of flavor compounds into the interior of the protein may disrupt hydrophobic interactions between protein segments, and thus destabilize the protein structure. Flavor ligands with reactive groups, such as aldehydes, can covalently bind to the ε-amino groups of lysine residues, change the net charge of the protein, and thus cause protein unfolding. Unfolding generally results in exposure of new hydrophobic sites for ligand binding. Because of these structural changes, Scatchard plots for protein are generally curvilinear. In the case of oligomeric proteins, such as soy proteins, conformational changes may involve both dissociation and unfolding of subunits. Denatured proteins generally exhibit a large number of binding sites with weak association constants. Methods for measuring flavor binding can be found in References 28 and 29.

5.5.4.2 Factors Influencing Flavor Binding

Since volatile flavors interact with hydrated proteins mainly via hydrophobic interactions, any factor that affects hydrophobic interactions or surface hydrophobicity of proteins will influence flavor

binding. Temperature has very little effect on flavor binding, unless there is significant thermal unfolding of the protein occurs. This is because the association process is primarily entropy driven, not enthalpy driven. Thermally denatured proteins exhibit increased ability to bind flavors; however, the binding constant is usually low compared to that of native proteins. The effects of salts on flavor binding are related to their salting-in and salting-out properties. Salting-in-type salts, which destabilize hydrophobic interactions, decrease flavor binding, whereas salting-out-type salts increase flavor binding.

The effect of pH on flavor binding is generally related to pH-induced conformational changes in proteins. Flavor binding is usually enhanced more at alkaline pH than at acid pH; this is because proteins tend to denature more extensively at alkaline pH than at acid pH. Breakage of protein disulfide bonds, which occurs at alkaline pH and causes unfolding of proteins, usually increases flavor binding. Proteolysis, which disrupts and decreases the number of hydrophobic regions in proteins, decreases flavor binding. This can be used as a way of removing off-flavors from oilseed proteins.

5.5.5 Viscosity

The consumer acceptability of several liquid and semisolid-type foods (e.g., gravies, soups, beverages, etc.) depends on the viscosity or consistency of the product. The viscosity of a solution relates to its resistance to flow under an applied force (or shear stress). For an ideal solution, the shear stress (i.e., force per unit area, F/A) is directly proportional to the shear rate (i.e., the velocity gradient between the layers of the liquid, $\mathrm{d}v/\mathrm{d}r$). This is expressed as

$$\frac{F}{A} = \eta \frac{\mathrm{d}v}{\mathrm{d}r} \tag{5.64}$$

The proportionality constant η is known as the viscosity coefficient. Fluids that obey the above expression are called Newtonian fluids.

The flow behavior of solutions is greatly influenced by solute type. Large molecular weight soluble polymers greatly increase viscosity even at very low concentrations. This again depends on several molecular properties such as size, shape, flexibility, and hydration. Solutions of randomly coiled macromolecules display greater viscosity than do solutions of compact folded macromolecules of same molecular weight.

Most macromolecular solutions, including protein solutions, do not display Newtonian behavior, especially at high protein concentrations. For these systems, the viscosity coefficient decreases when the shear rate increases. This behavior is known as *pseudoplastic* or *shear-thinning*, and follows the relationship:

$$\frac{F}{A} = m\left(\frac{\mathrm{d}v}{\mathrm{d}r}\right)^n \tag{5.65}$$

where m is the consistency coefficient and n is an exponent known as the "flow behavior index." The pseudoplastic behavior of protein solutions arises because of the tendency of protein molecules to orient their major axes in the direction of flow. Dissociation of weakly held dimers and oligomers into monomers also contribute to shear-thinning. When shearing or flow is stopped, the viscosity may or may not return to the original value depending on the rate of relaxation of the protein molecules to random orientation. Solutions of fibrous proteins, for example, gelatin and actomyosin, usually remain oriented, and thus do not quickly regain their original viscosity. On the other hand, solutions of globular proteins, for example, soy proteins and whey proteins, rapidly regain their viscosity when flow is stopped. Such solutions are called *thixotropic*.

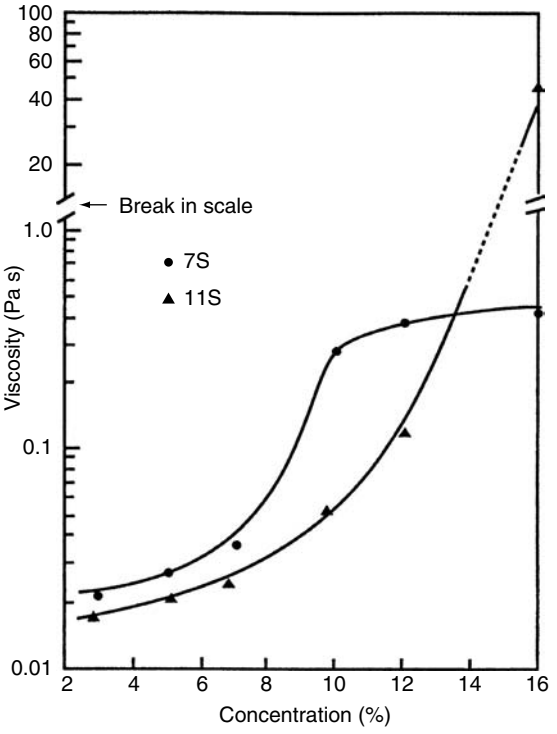


FIGURE 5.28 Effect of concentration on viscosity (or consistency index) of 7S and 11S soy protein solutions at 20°C. (From Rao, M. A. et al. 1986. In *Food Engineering and Process Applications* (Le Maguer, M. and P. Jelen, Eds.), Elsevier Applied Sci., New York, pp. 39–48.)

The viscosity (or consistency) coefficient of most protein solutions follows an exponential relationship with protein concentration because of both protein–protein interactions and interactions between the hydration spheres of protein molecules. An example involving soy protein fractions is shown in Figure 5.28 [104]. At high protein concentrations or in protein gels, where protein–protein interactions are numerous and strong, proteins display plastic viscoelastic behavior. In these cases, a specific amount of force, known as "yield stress," is required to initiate flow.

The viscosity behavior of proteins is a manifestation of complex interactions among several variables, including size, shape, protein–solvent interactions, hydrodynamic volume, and molecular flexibility in the hydrated state. When dissolved in water, proteins absorb water and swell. The volume of the hydrated molecules is much larger than their unhydrated volume. The protein-associated water induces long-range effects on the flow behavior of the solvent. The dependence of viscosity on shape and size of protein molecules follows the relationship:

$$\eta_{sp} = \beta C(\bar{\upsilon}_2 + \delta_1 \bar{\upsilon}_1) \tag{5.66}$$

where η_{sp} is specific viscosity, β is the shape factor, C is concentration, $\bar{\upsilon}_2$ and $\bar{\upsilon}_1$ are specific volumes of unhydrated protein and solvent, respectively, and δ_1 is grams of water bound per gram of protein. Here, $\bar{\upsilon}_2$ is also related to molecular flexibility; the greater the specific volume of the protein, the greater is its flexibility.

The viscosity of dilute protein solutions is expressed in several ways. *Relative viscosity* η_{rel} refers to the ratio of viscosity of the protein solution to that of the solvent. It is measured in an

Ostwald–Fenske type capillary viscometer, and is expressed as

$$\eta_{\text{rel}} = \frac{\eta}{\eta_0} = \frac{\rho t}{\rho_0 t_0} \tag{5.67}$$

where ρ and ρ_0 are densities of protein solution and solvent, respectively, and t and t_0 are times of flow for a given volume of protein solution and solvent, respectively, through the capillary. Other forms of expressing viscosity can be obtained from the relative viscosity. *Specific viscosity* is defined as

$$\eta_{\text{sp}} = \eta_{\text{rel}} - 1 \tag{5.68}$$

Reduced viscosity is

$$\eta_{\text{red}} = \frac{\eta_{\text{sp}}}{C} \tag{5.69}$$

where C is the protein concentration, and *intrinsic viscosity* is

$$[\eta] = \text{Lim}\frac{\eta_{\text{sp}}}{C} \tag{5.70}$$

The intrinsic viscosity, $[\eta]$, is obtained by extrapolating a plot of reduced viscosity vs. protein concentration to zero protein concentration (Lim). Since protein–protein interactions are nonexistent at infinite dilution, intrinsic viscosity accurately depicts the effects of shape and size on the flow behavior of individual protein molecules. Changes in the hydrodynamic shape of proteins that result from heat and pH treatments can be studied by measuring their intrinsic viscosities.

5.5.6 Gelation

A gel is an intermediate phase between a solid and a liquid. Technically, it is defined as "a substantially diluted system that exhibits no steady state flow" [43]. It is made up of polymers cross-linked via either covalent or noncovalent bonds to form a network that is capable of entrapping water and other small molecular-weight substances (see Chapter 13).

Protein gelation refers to transformation of a protein from the "sol" state to a "gel-like" state. Heat, enzymes, or divalent cations under appropriate conditions facilitate this transformation. All these agents induce formation of network structure; however, the types of covalent and noncovalent interactions involved, and the mechanism of network formation can differ considerably.

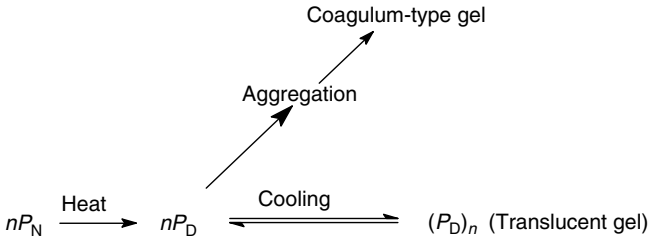
Most food protein gels are prepared by heating a moderately concentrated protein solution. In this mode of gelation, the protein in a "sol" state is first transformed into a "progel" state by denaturation. In the "sol" state, the number of noncovalent bonding groups available in proteins for network structure formation is limited. The progel state however is usually a viscous liquid state in which some degree of protein denaturation and polymerization has already occurred. Also, in the progel state, a critical number of functional groups, such as hydrogen bonding and hydrophobic groups that can form intermolecular noncovalent bonds, become exposed so that the second stage, formation of a protein network, can occur. The conversion of sol to progel is irreversible because many protein–protein interactions occur between the unfolded molecules. When the progel is cooled to ambient or refrigeration temperature, the decrease in the thermal kinetic energy facilitates formation of stable noncovalent bonds among exposed functional groups of the various molecules and this constitutes gelation.

The interactions involved in network formation are primarily hydrogen bonds, and hydrophobic and electrostatic interactions. The relative contributions of these forces vary with the type of protein, heating conditions, the extent of denaturation, and environmental conditions. Hydrogen bonding

and hydrophobic interactions contribute more than electrostatic interactions to network formation except when multivalent ions are involved in cross-linking. Since proteins generally carry a net charge, electrostatic repulsion occurs among protein molecules and this is not usually conducive to network formation. However, charged groups are essential for maintaining protein–water interactions and water-holding capacity of gels.

Gel networks that are sustained primarily by noncovalent interactions are thermally reversible; that is, upon reheating they will melt to a progel state, as is commonly observed with gelatin gels. This is especially true when hydrogen bonds are the major contributors to the network. Since hydrophobic interactions are strong at elevated temperatures, gel networks formed primarily by hydrophobic interactions are thermally irreversible, for example, egg white gels. Proteins that contain both cysteine and cystine groups can undergo polymerization via sulfhydryl–disulfide interchange reactions during heating and form a continuous covalent network upon cooling. Such gels are usually thermally irreversible. Examples of gels of this type are ovalbumin, β-lactoglobulin, and whey protein gels.

Proteins form two types of gels, that is, coagulum (opaque) gels and translucent gels. The type of gel formed by a protein is dictated by its molecular properties and solution conditions. Proteins containing large amounts of nonpolar amino acid residues undergo hydrophobic aggregation upon denaturation.



(5.71)

P_N is native state, P_D is unfolded state, and n is the number of protein molecules taking part in cross-linking.

These insoluble aggregates then randomly associate and set into an irreversible coagulum-type gel. Since the rate of aggregation and network formation is faster than the rate of denaturation, proteins of this type readily set into a gel network even while being heated. The opaqueness of these gels is due to light scattering caused by the unordered (isotropic) network of insoluble protein aggregates. Coagulum-type gels are generally weak and are prone to syneresis.

Proteins that contain small amounts of nonpolar amino acid residues form soluble complexes upon denaturation. Since the rate of association of these soluble complexes is slower than the rate of denaturation, and the gel network is predominantly formed by hydrogen bonding interactions, they often do not set into a gel until heating followed by cooling has occurred (typically 8–12% protein concentration is used). Upon cooling, the slow rate of association of the soluble complexes facilitates formation of an ordered translucent gel network.

At the molecular level, coagulum-type gels tend to form when the sum of Val, Pro, Leu, Ile, Phe, and Trp residues of the protein exceeds 31.5 mol% [116]. Those that contain <31.5 mol% of the above hydrophobic residues usually form translucent gels when water is the solvent. However, this dictum is not obeyed when salt solutions are used as the solvents. For example, the hydrophobic amino acid content of β-lactoglobulin is 32 mol%, yet it forms a translucent gel in water. However, when NaCl is included, it forms a coagulum-type gel even when the salt concentration is as low as 50 mM. This occurs because of charge neutralization by NaCl, which promotes hydrophobic aggregation upon heating. Thus, the balance between attractive hydrophobic interactions and repulsive electrostatic interactions fundamentally controls gelation mechanism and the gel appearance. These two forces in effect control the balance between protein–protein and protein–solvent interactions in a gelling

system. If the former is much greater than the latter, a precipitate is likely to form. If protein–solvent interactions predominate, the system may not gel. A coagulum gel or a translucent gel results when the magnitude of hydrophobic and hydrophilic forces are somewhere in-between these two extremes.

Protein gels are highly hydrated systems, containing up to 98% water in some cases. The water entrapped in these gels has chemical potential (activity) similar to that in dilute aqueous solutions, but lacks fluidity and cannot be easily expressed out. The mechanism by which liquid water can be held in a semisolid state in gels is not well understood. However, the fact that translucent gels, formed primarily by hydrogen bonding interactions, hold more water than coagulum-type gels and are less prone to syneresis, suggests that much of the water is hydrogen bonded to C═O and N—H groups of the peptide bonds, is associated with charged groups in the form of hydration shells, and/or exists in extensively hydrogen-bonded ice-like water–water networks. It is also possible that within the restricted environment of the microstructure of the gel network, water may exist as a hydrogen-bonding cross-linker between C═O and N—H groups of peptide segments (see Chapter 2). This may restrict the flowability of water within each cell, the more so as the cell size decreases. It is also likely that some water may be held as capillary water in the pores of the gel structure, especially in coagulum gels.

The stability of a gel network against thermal and mechanical forces is dependent on the number and types of cross-links formed per monomer chain. Thermodynamically, a gel network would be stable only when the sum of the interaction energies of a monomer in the gel network is greater than its thermal kinetic energy. This is dependent on several intrinsic (such as the size, net charge, etc.) and extrinsic factors (such as pH, temperature, ionic strength, etc.). The square root of the hardness of protein gels exhibits a linear relationship with molecular weight [125]. Globular proteins with molecular weight $<$23,000 Da cannot form a heat-induced gel at any reasonable protein concentration, unless they contain at least one free sulfhydryl group or a disulfide bond. The sulfhydryl groups and disulfide bonds facilitate polymerization, and thus increase the effective molecular weight of polypeptides to $>$23,000 Da. Gelatin preparations with effective molecular weights of $<$20,000 Da cannot form a gel.

Another critical factor is protein concentration. To form a self-standing gel network, a minimum protein concentration, known as least concentration endpoint (LCE), is required [50]. The LCE is 8% for soy proteins, 3% for egg albumin, and about 0.6% for gelatin. Above this minimum concentration, the relationship between gel strength, G, and protein concentration, C, usually follows a power law:

$$G \propto (C - C_0)^n \tag{5.72}$$

where C_0 is the LCE. For proteins, the value of n varies from 1 to 2.

Several environmental factors, such as pH, salts, and other additives also affect gelation of proteins. At or near isoelectric pH, proteins usually form coagulum-type gels. At extremes of pH, weak gels are formed because of strong electrostatic repulsion. The optimum pH for gel formation is about 7–8 for most proteins.

Formation of protein gels can sometimes be facilitated by limited proteolysis. A well-known example is cheese. Addition of chymosin (rennin) to casein micelles in milk results in the formation of a coagulum-type gel. This is achieved by cleavage of κ-casein, a micelle component, causing release of a hydrophilic portion, known as the glycomacropeptide. The remaining so-called para-casein micelles possess a highly hydrophobic surface that facilitates formation of a weak gel network.

Enzymic cross-linking of proteins at room temperature can also result in formation of a gel network. Transglutaminase is the enzyme often used to prepare these gels. This enzyme catalyses formation of ε-(γ-glutamyl)lysyl cross-links between the glutamine and lysyl groups of protein molecules [91]. Using this enzymic cross-linking method, highly elastic and irreversible gels can be formed even at low protein concentration.

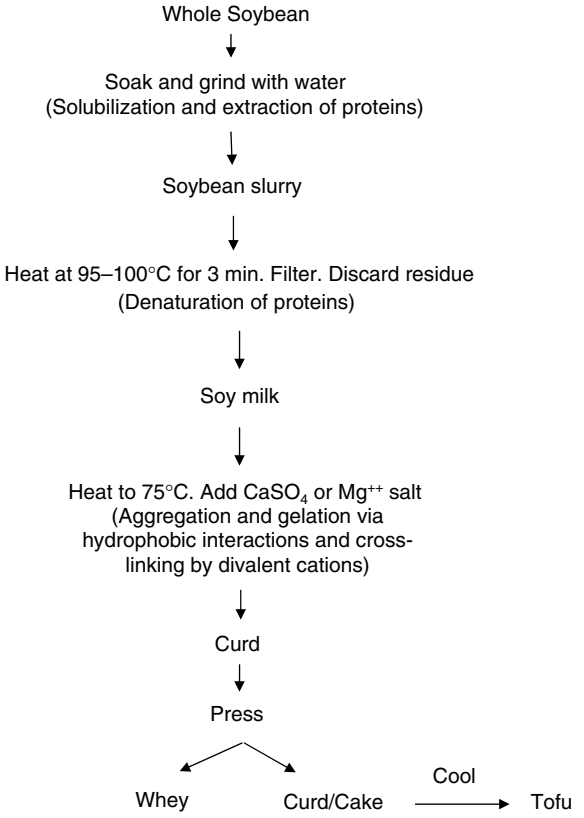


FIGURE 5.29 A typical commercial process for tofu manufacture.

Divalent cations, such as Ca^{2+} and Mg^{2+}, can also be used to form protein gels. These ions form cross-links between negatively charged groups of protein molecules. A good example of this type of gel is tofu from soy proteins. Alginate gels also can be formed in this manner. A general method for making tofu is outlined in Figure 5.29.

5.5.7 Texturization

Texturization connotes transformation of a protein from a globular state to a fibrous physical structure that has meat-like mouthfeel characteristics. The various functional properties that texturized protein products are expected to possess include chewiness, elasticity, softness, and juiciness. Vegetable proteins are often the preferred protein source for texturization, primarily because they lack other desirable functional properties that proteins of animal origin display. Textured vegetable proteins are manufactured using two different processes, namely *spun-fiber texturization* and *extrusion texturization*.

5.5.7.1 Spun-Fiber Texturization

In this process, a highly concentrated (~20% w/v) soy protein isolate solution is adjusted to pH 12–13 and aged until the viscosity of the solution increases to 50,000–100,000 centipoise as a result of protein denaturation and certain alkali-induced cross-linking reactions. This highly viscous "dope" is then pumped through a spinneret, a device with a plate containing thousands of micron-size holes. The fibrous extrudate is passed through a bath containing phosphoric acid and salt at pH 2.5. The

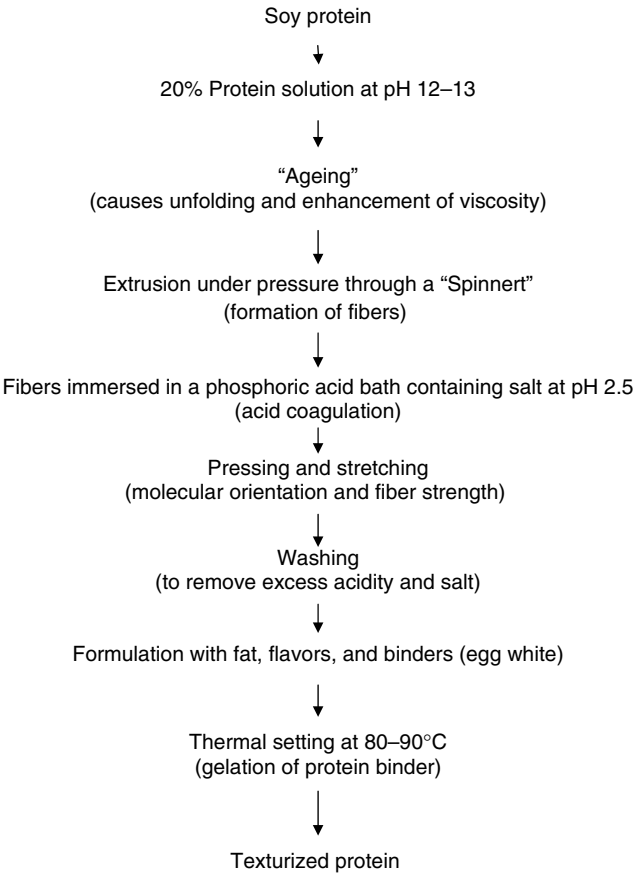


FIGURE 5.30 A typical spun-fiber process for texturization of soy proteins.

protein coagulates instantaneously in this bath and becomes a fibrous mass. The fiber is then "towed" through steel rolls where it is compressed and stretched to enhance its strength. The fiber is then passed through a washing bath where excess acidity and salt are removed. The washed fibers are then passed through a series of tanks containing fat, flavors, colors, and binders depending on the final product. The fiber is then heated at 80–90°C to induce gelation of the binder protein. Egg white is often used as a binder because of its excellent heat coagulation properties. The final product is dried and sized. A process flow chart for the spun-fiber texturization process is outlined in Figure 5.30.

5.5.7.2 Extrusion Texturization

In this process, defatted soy flour or soy protein concentrate with high protein solubility index (PSI) is conditioned with steam and the moisture content is adjusted to 20–25%. This solid mass is then fed to an extruder, which is mainly a rotating screw housed in a tapered cylindrical barrel in which the space between the screw and the barrel decreases progressively along the screw axis. As the protein mass advances through the screw, it is rapidly heated to 150–180°C. This high temperature and the progressive build up of pressure as the mass moves down the screw results in pressure-cooking, and as a result the protein mass melts and the proteins are denatured. In technical terms this is known as thermoplastic melt. The denatured proteins become aligned in fiber form as the mass moves through the screw. When the mass exits the die, sudden release of pressure evaporates water and puffs the product. Adjusting the pressure and temperature can control puffing. If a dense product is desired, the mass is

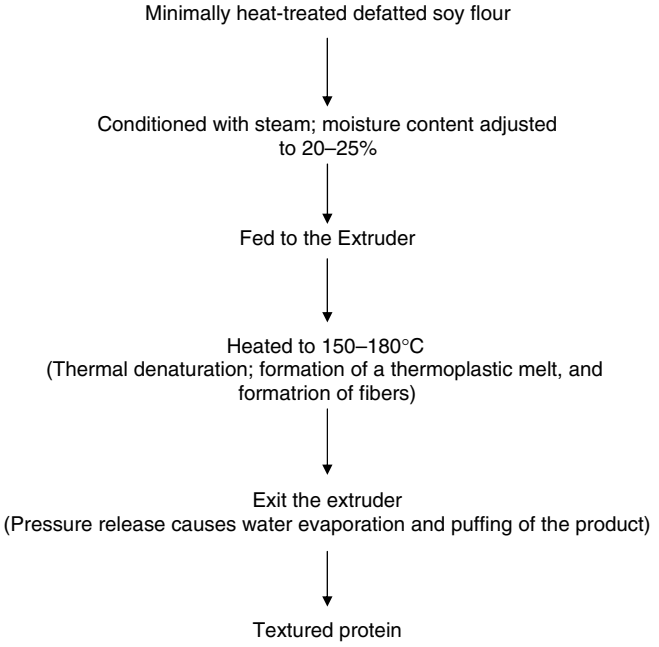


FIGURE 5.31 Extrusion texturization of soy flour.

cooled before it exits the die. The extrudate is then cut to pieces and processed further depending on its use. A general process flow chart for extrusion texturization of proteins is shown in Figure 5.31.

The general principles involved in both these methods are thermal or alkaline denaturation of proteins, realignment of the denatured proteins in the form of a fibrous network, binding of the fibers using a protein binder, and flavoring of the final product. Texturized vegetable proteins are increasingly being used as meat extenders in comminuted meat products (meat patties, sauces, burgers, etc.) and as meat analogs or "imitation meat."

5.5.8 Dough Formation [79,80,115]

When a mixture of wheat flour and water (about 3:1 ratio) is kneaded, it forms a viscoelastic dough suitable for making bread and other bakery products. These unusual dough characteristics are mainly attributable to the proteins in wheat flour.

Wheat flour contains several soluble and insoluble protein fractions. The soluble proteins, comprising about 20% of the total proteins, are primarily albumin and globulin type enzymes and certain minor glycoproteins. These proteins do not contribute to the dough-forming properties of wheat flour. The major storage protein of wheat is gluten. Gluten is a heterogeneous mixture of proteins, mainly gliadins and glutenins, with limited solubility in water. When mixed with water, gluten forms viscoelastic dough capable of entrapping gas during fermentation.

Gluten has a unique amino acid composition, with Gln and Pro accounting for more than 40% of the amino acid residues (Table 5.18). The low water solubility of gluten is attributable to its low content of Lys, Arg, Glu, and Asp residues, which together amount to $<10\%$ of the total amino acid residues. About 30% of gluten's amino acid residues are hydrophobic, and the residues contribute greatly to its ability to form protein aggregates by hydrophobic interactions, and to bind lipids and other nonpolar substances. The high glutamine and hydroxyl amino acid ($\sim 10\%$) contents of gluten are responsible for its water binding properties. In addition, hydrogen bonding between glutamine and hydroxyl residues of gluten polypeptides contributes to its cohesion–adhesion properties. Cysteine and cystine residues account for 2–3 mol% of gluten's total amino acid residues. During formation of

TABLE 5.18
Amino Acid Composition of Glutenin and Gliadin

Amino Acid	Glutenin (mol%)	Gliadin (mol%)
Cys	2.6	3.3
Met	1.4	1.2
Asp	3.7	2.8
Thr	3.4	2.4
Ser	6.9	6.1
Glx[a]	28.9	4.6
Pro	11.9	16.2
Gly	7.5	3.1
Ala	4.4	3.3
Val	4.8	4.8
Ile	3.7	4.3
Leu	6.5	6.9
Tyr	2.5	1.8
Phe	3.6	4.3
Lys	2.0	0.6
His	1.9	1.9
Arg	3.0	2.0
Trp	1.3	0.4

[a] Glx corresponds to mixture of Glu and Gln. Most of Glx in wheat protein is in the form of Gln (37).

the dough, these residues undergo sulfhydryl–disulfide interchange reactions, resulting in extensive polymerization of gluten proteins [115].

Several physical and chemical transformations occur during mixing and kneading of a mixture of wheat flour and water. Under the applied shear and tensile forces, gluten proteins absorb water and partially unfold. The partial unfolding of protein molecules facilitates hydrophobic interactions and sulfhydryl–disulfide interchange reactions, which result in formation of thread-like polymers. These linear polymers in turn are believed to interact with each other, presumably via hydrogen bonding, hydrophobic associations, and disulfide cross-linking, to form a sheet-like film capable of entrapping gas. Because of these transformations in gluten, the resistance of the dough increases with time until a maximum is reached and this is followed by a decrease in resistance indicative of a breakdown in the network structure. The breakdown involves alignment of polymers in the direction of shear and some scission of disulfide cross-links, which reduces the polymer size. The time it takes to reach maximum dough strength (R_{max}) during kneading is used as a measure of wheat quality for bread making—a longer time indicating better quality.

The viscoelasticity of wheat dough is related to the extent of sulfhydryl–disulfide interchange reactions. This view is supported by the fact that when reductants, such as cysteine, or sulfhydryl blocking agents, such as *N*-ethylmaleimide, are added to dough, viscoelasticity decreases greatly. On the other hand, addition of oxidizing agents, such as iodates and bromates, increase the elasticity of the dough. This implies that wheat gluten rich in SH and S—S groups might possess superior bread making qualities, but this relationship is unreliable. Thus, interactions other than disulfide cross-links, such as hydrogen bonding and hydrophobic interactions, also play a vital role in viscoelasticity of wheat dough.

Differences in bread-making qualities of different wheat cultivars may be related to differences in the composition of gluten itself. As mentioned earlier, gluten is made up of gliadins and glutenins. Gliadins are comprised of four groups, namely α-, β-, γ-, and ω-gliadins. In gluten these exist as single polypeptides with molecular weights ranging from 30,000 to 80,000 Da. Gliadins contain even number of cysteine residues. They exist as intramolecular disulfide bonds. The disulfide bonds are

buried in the interior of the protein so that they do not take part in sulfhydryl–disulfide interchange reactions with other proteins. The disulfide bonds appear to remain as intramolecular disulfides during dough making. Thus, dough made from isolated gliadins and starch is viscous but not viscoelastic.

Glutenins, on the other hand, are heterogeneous polypeptides with molecular weights ranging from 12,000 to 130,000 Da. These are further classified into high molecular weight (MW > 90,000, HMW) and low molecular weight (MW < 90,000, LMW) glutenins. In gluten, these glutenin polypeptides are present as polymers joined by disulfide cross-links, with molecular weights ranging into the millions. Because of their ability to polymerize extensively via sulfhydryl–disulfide interchange reactions, glutenins contribute greatly to the elasticity of dough. Some studies have shown a significant positive correlation between HMW glutenin content and bread making quality in some wheat varieties [10]. Available information indicates that a specific pattern of disulfide cross-linked association between LMW and HMW glutenins in gluten structure may be far more important to bread quality than the amount of HMW protein. For example, association/polymerization among LMW glutenins gives rise to a structure similar to that formed by HMW gliadin. This type of structure contributes to viscosity of the dough, but not to elasticity. In contrast, the dough elasticity increases when LMW glutenins cross-link to HMW glutenins via disulfide cross-links (in gluten). It is possible that in good quality wheat varieties, more of the LMW glutenins may polymerize to HMW, whereas in poor quality wheat varieties, most of the LMW glutenins may polymerize among themselves. These differences in associated states of glutenins in gluten of various wheat varieties may be related to differences in their conformational properties, such as surface hydrophobicity, and reactivity of sulfhydryl and disulfide groups.

In summary, hydrogen bonding among amide and hydroxyl groups, hydrophobic interactions, and sulfhydryl–disulfide interchange reactions all contribute to the development of the unique viscoelastic properties of wheat dough. However, culmination of these interactions into good dough making properties may depend on the structural properties of each protein and the proteins with which it associates in the overall gluten structure.

Because polypeptides of gluten, especially the glutenins, are rich in proline, they have very little ordered secondary structure. Whatever ordered structure initially exists in gliadins and glutenins is lost during mixing and kneading. Therefore, no additional unfolding occurs during baking.

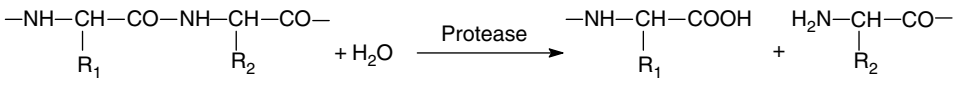
Supplementation of wheat flour with albumin and globulin type proteins, for example, whey proteins and soy proteins, adversely affects the viscoelastic properties and baking quality of the dough. These proteins decrease bread volume by interfering with formation of the gluten network. Addition of phospholipids or other surfactants to dough counters the adverse effects of foreign proteins on loaf volume. In this case, the surfactant/protein film compensates for the impaired gluten film. Although this approach results in acceptable loaf volume, the textural and sensory qualities of the bread are less desirable than normal.

Isolated gluten is sometimes used as a protein ingredient in nonbakery products. Its cohesion–adhesion properties make it an effective binder in comminuted meat and surimi-type products.

5.6 PROTEIN HYDROLYSATES

Partial hydrolysis of proteins using proteolytic enzymes is one of the strategies for improving the functional properties. Functional properties such as solubility, dispersibility, foaming, and emulsification can be potentially improved by limited proteolysis of proteins. Protein hydrolysates have many uses in speciality foods such as geriatric foods, nonallergenic infant formula, sports drinks, and diet foods. Because protein hydrolysates can be readily digested, they are particularly useful in infant formula and geriatric foods.

Proteolysis denotes enzymatic hydrolysis of peptide bonds in proteins.



(5.73)

In this reaction, for every peptide bond cleaved by the enzyme, one mole each of carboxyl group and amino group is liberated. When the reaction is allowed to completion, the final product is a mixture of all constituent amino acids of the protein. Incomplete proteolysis results in liberation of a mixture of polypeptides from the original protein. The functional properties of the protein hydrolysate is dependent upon the degree of hydrolysis (DH) and the physicochemical properties, that is, size, solubility, and so forth, of the polypeptides in the hydrolysate.

The DH is defined as the fraction of peptide bonds cleaved and it is often expressed as percentage:

$$\%\mathrm{DH} = \frac{n}{n_{\mathrm{T}}} \times 100 \tag{5.74}$$

where n_{T} is the total number of moles of peptide bonds present in one mole of protein and n is the number of moles of peptide bonds cleaved per of mole of protein. When molar mass of a protein is not known or the protein sample is a mixture of various proteins, n and n_{T} are expressed as the number of peptide bonds per gram of protein.

The DH is generally monitored using the pH-Stat method. The principle behind this method is that when a peptide bond is hydrolyzed, the newly formed carboxyl group completely ionizes at pH > 7, which releases H^+ ion. As a result, the pH of the protein solution progressively decreases with time of hydrolysis. In the pH range 7–8, the number of moles of H^+ ion released is equivalent to the number of moles of peptide bonds hydrolyzed. In the pH-Stat method, the pH of the protein solution is maintained at a constant pH by titrating with NaOH. The number of moles of NaOH consumed during proteolysis is equivalent to the number of moles of peptide bonds cleaved.

Several proteases can be potentially used for preparing protein hydrolysates. Some of these proteases are site-specific enzymes (Table 5.19). Because of their specificity, the types of polypeptide fragments released in the hydrolysate differ between proteases. Alcalase from *Bacillus licheniformis* is a major commercial enzyme used in the manufacture of protein hydrolysate. This enzyme belongs to a family of subtilisins, which are serine proteases.

5.6.1 Functional Properties

The functional properties of protein hydrolysates depend on the type of enzymes used in their preparation. This is primarily because of differences in the size and other physicochemical properties of the polypeptides released during hydrolysis. Generally, solubility of most proteins improves after hydrolysis regardless of the enzyme used. The greater the DH, the higher is the solubility. However,

TABLE 5.19
Specificity of Various Proteases

Protease	Type	Specificity
Elastase	Endoproteinase	Ala—aa; Gly—aa
Bromelain	Endoproteinase	Ala—aa; Tyr—aa
Trypsin	Endoproteinase	Lys—aa; Arg—aa
Chymotrypsin	Endoproteinase	Phe—aa; Trp—aa; Tyr—aa
Pepsin	Endoproteinase	Leu—aa; Phe—aa
V-8 protease	Endoproteinase	Asp—aa; Glu—aa
Thermolysin	Endoproteinase	aa—Phe; aa—Leu
Alcalase	Endoproteinase	Nonspecific
Papain	Endoproteinase	Lys—aa; Arg—aa; Phe—aa; Gly—aa
Prolylendopeptidase	Endoproteinase	Pro—aa
Subtilisin A	Endoproteinase	Nonspecific

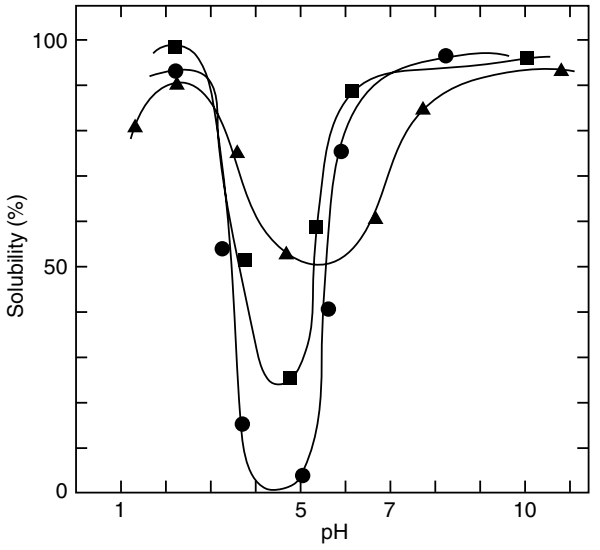


FIGURE 5.32 pH-solubility profiles of native casein and of *Staphylococcus aureus* V-8 protease-modified casein. The solubility was expressed as percent of total protein in solution. ●, native casein; ■, 2% DH; ▲, 6.7% DH. (From Adler-Nissen, J. 1979. *J. Agric. Food Chem.* **27**:1256–1260.)

the net increase in solubility depends on the type of enzyme used. Shown in Figure 5.32 [2] is the pH-solubility profile of casein before and after hydrolysis with V-8 protease. It should be noted that the solubility of casein at its isoelectric pH is significantly increased after partial hydrolysis. This type of behavior is also observed with other proteins. Higher protein solubility is particularly important in acidic protein drinks in which precipitation and sedimentation is undesirable.

Since solubility of a protein is essential for its foaming and emulsifying properties, partially hydrolyzed proteins generally show improved foaming and emulsifying properties. However, this improvement is dependent on the type of enzyme used and the DH. Generally, the foaming and emulsifying capacity improve up to DH $< 10\%$ and decrease at DH $> 10\%$. On the other hand, the stabilities of foams and emulsions made with protein hydrolysates are generally lower than that of the intact protein. One of the reasons for this is the inability of the short polypeptides to form a cohesive viscoelastic film at the air–water and oil–water interfaces.

Protein hydrolysates generally do not form heat-induced gels. One exception is gelatin. Gelatin is produced from collagen by acid or alkaline hydrolysis. Gelatin is a heterogeneous mixture of polypeptides. The weight-average-molecular weight of polypeptides in a gelatin sample depends on the DH. This profoundly affects their gel strength. The higher the weight-average-molecular weight, the higher is the gel strength. Gelatin samples with weight-average-molecular weight $<$20,000 Da do not form gels at all gelatin concentration [43]. The gelling properties of commercial gelatin products are expressed in terms of bloom rating measured using a bloom gelometer. The bloom rating is defined as the weight in grams required for driving a plunger of a gelometer 4 cm into a 6.67% (w/v) gelatin gel that has been incubated for 17 h in a water bath at 10°C. Table 5.20 shows the bloom rating requirements for various types of gelatin-based food products.

5.6.2 Allergenicity

Several food proteins, including cow's milk, soy proteins, gluten, egg proteins, and peanut proteins, cause severe allergic reactions in children and adults. Among the population who are allergic to milk proteins, about 60% are allergic to caseins, 60–80% to β-lactoglobulin, and 50% to α-lactalbumin [1]. However, hydrolysates of these proteins possess lower allergenicity than their native counterparts.

TABLE 5.20
Bloom Rating Requirements for Some Gelatin-Based Food Products

Product	Bloom Rating (g)	Concentration Used in Food (%)
Jelly beans	220	7–8
Fruit jelly	100–120	10–12
Marshmallow	220	2–3
Lozenges	50–100	1

Allergenicity of intact proteins arises because of the presence of antigenic sites (epitopes) that bind to immunoglobulin E (IgE). In protein hydrolysates, the epitopes are destroyed by proteolytic cleavage. For instance, hydrolysis of casein up to 55% DH using pancreatin (mixture of pancreatic enzymes) decreases its allergenicity by about 50% [81]. Similarly, whey protein hydrolysates with 12.9–16.1% DH fail to produce allergenic reaction when tested in guinea pigs sensitized with intact whey proteins [97]. Thus, protein hydrolysates are the preferred source of protein/amino acid for infants and children who are predisposed or at high risk of developing allergic reaction to food proteins.

The net reduction in allergenicity of protein hydrolysates depends on the type of protease used. Nonspecific proteases or a mixture of proteases are more effective than a site-specific protease in reducing the allergenicity of proteins. The DH also plays a role: the higher the DH, the greater is the reduction of allergenicity. For these reasons, the efficacy of proteases in reducing allergenicity of a protein is often expressed as allergenicity reduction index (ARI). ARI is defined as the ratio of % reduction in allergenicity to %DH.

5.6.3 Bitter Peptides

One of the most undesirable properties of protein hydrolysates is their bitter flavor. The bitterness emanates from certain peptides released during hydrolysis. There is ample evidence that bitterness of peptides is related to hydrophobicity. Peptides with a mean residue hydrophobicity of <1.3 kcal/mol are not bitter (see Chapter 10). On the other hand, peptides with a mean residue hydrophobicity of >1.4 kcal/mol are bitter [3]. In this case, often the mean residue hydrophobicity of the peptides is calculated using the free energies of transfer of amino acid residues from ethanol to water (see Table 10.1). Formation of bitter peptides in protein hydrolysates depends on the amino acid composition and sequence, and the type of enzymes used. Hydrolysates of highly hydrophobic proteins such as casein, soy proteins, and corn protein (zein) are very bitter, whereas hydrolysates of hydrophilic proteins such as gelatin are less bitter. Caseins and soy proteins hydrolyzed with several commercial proteases produce several bitter peptides. The bitterness can be reduced or eliminated by using a mixture of endo- and exopeptidases, which further breakdown bitter peptides into fragments that have <1.3 kcal/mol mean residue hydrophobicity.

5.7 NUTRITIONAL PROPERTIES OF PROTEINS

Proteins differ in their nutritive value. Several factors, such as essential amino acids content and digestibility, contribute to these differences. The daily protein requirement, therefore, depends on the type and composition of proteins in a diet.

5.7.1 Protein Quality

The "quality" of a protein is related mainly to its essential amino acids content and digestibility. High quality proteins are those that contain all the essential amino acids at levels greater than the FAO/WHO/UNU [40] reference levels, and a digestibility comparable to or better than those of egg white or milk proteins. Animal proteins are of better "quality" than plant proteins.

Proteins of major cereals and legumes are often deficient in at least one of the essential amino acids. While proteins of cereals, such as rice, wheat, barley, and maize are very low in lysine and rich in methionine, those of legumes and oilseeds are deficient in methionine and rich or adequate in lysine. Some oilseed proteins, such as peanut protein, are deficient in both methionine and lysine contents. The essential amino acids whose concentrations in a protein are below the levels of a reference protein are termed *limiting amino acids.* Adults consuming only cereal proteins or legume proteins have difficulty maintaining their health; children below 12 years of age on diets containing only one of these protein sources cannot maintain a normal rate of growth. The essential amino acid contents of various food proteins are listed in Table 5.21 [35,40].

Both animal and plant proteins generally contain adequate or more than adequate amounts of His, Ile, Leu, Phe + Tyr, and Val. These amino acids usually are not limiting in staple foods. More often, Lys, Thr, Trp, and the sulfur containing amino acids are the limiting amino acids. The nutritional quality of a protein that is deficient in an essential amino acid can be improved by mixing it with another protein that is rich in that essential amino acid. For example, mixing of cereal proteins with legume proteins provides a complete and balanced level of essential amino acids. Thus, diets containing appropriate amounts of cereals and legumes (pulses) and otherwise nutritionally complete are often adequate to support growth and maintenance. A poor quality protein also can be nutritionally improved by supplementing it with essential free amino acids that are under-represented. Supplementation of legumes with Met and cereals with Lys usually improves their quality.

The nutritional quality of a protein or protein mixture is ideal when it contains all of the essential amino acids in proportions that produce optimum rates of growth and/or optimum maintenance capability. The ideal essential amino acid patterns for children and adults are given in Table 5.22 [108]. However, because actual essential amino acid requirements of individuals in a given population vary depending on their nutritional and physiological status, the essential amino acid requirements of preschool children (age 2–5) are generally recommended as a safe level for all age groups [39].

Overconsumption of any particular amino acid can lead to "amino acid antagonism" or toxicity. Excessive intake of one amino acid often results in an increased requirement for other essential amino acids. This is due to competition among amino acids for absorption sites on the intestinal mucosa. For example, high levels of Leu decrease absorption of Ile, Val, and Tyr even if the dietary levels of these amino acids are adequate. This leads to an increased dietary requirement for the latter three amino acids. Overconsumption of other essential amino acids also can inhibit growth and induce pathological conditions.

5.7.2 Digestibility

Although the content of essential amino acids is the primary indicator of protein quality, true quality also depends on the extent to which these amino acids are utilized in the body. Thus, digestibility (bioavailability) of amino acids can affect the quality of proteins. Digestibilities of various proteins in humans are listed in Table 5.23 [40]. Food proteins of animal origin are more completely digested than those of plant origin. Several factors affect digestibility of proteins.

5.7.2.1 Protein Conformation

The structural state of a protein influences its hydrolysis by proteases. Native proteins are generally less completely hydrolyzed than partially denatured ones. For example, treatment of phaseolin (a protein from kidney beans) with a mixture of proteases results only in limited cleavage of the protein resulting in liberation of a 22,000 Da polypeptide as the main product. When heat-denatured phaseolin is treated under similar conditions, it is completely hydrolyzed to amino acids and dipeptides. Generally, insoluble fibrous proteins and extensively denatured globular proteins are difficult to hydrolyze.

TABLE 5.21
Essential Amino Acid Contents and Nutritional Value of Proteins from Various Sources (mg/g Protein)

	Protein Source												
Property (mg/g Protein)	**Egg Milk**	**Cow's**	**Beef**	**Fish**	**Wheat**	**Rice**	**Maize**	**Barley**	**Soybean**	**Field (Boiled)**	**Pea**	**Peanut Bean**	**French**
Amino acid concentration (mg/g protein)													
His	22	27	34	35	21	21	27	20	30	26	26	27	30
Ile	54	47	48	48	34	40	34	35	51	41	41	40	45
Leu	86	95	81	77	69	77	127	67	82	71	70	74	78
Lys	70	78	89	91	23[a]	34[a]	25[a]	32[a]	68	63	71	39[a]	65
Met + Cys	57	33	40	40	36	49	41	37	33	22[b]	24[b]	32	26
Phe + Tyr	93	102	80	76	77	94	85	79	95	69	76	100	83
Thr	47	44	46	46	28	34	32[b]	29[b]	41	33	36	29[b]	40
Trp	17	14	12	11	10	11	6[b]	11	14	8[a]	9[a]	11	11
Val	66	64	50	61	38	54	45	46	52	46	41	48	52
Total essential amino acids	512	504	480	485	336	414	422	356	466	379	394	400	430
Protein content (%)	12	3.5	18	19	12	7.5	—	—	40	32	28	30	30
Chemical score (%) (based on FAO/WHO, 1985 pattern)	100	100	100	100	40	59	43	55	100	73	82	67	—
PER	3.9	3.1	3.0	3.5	1.5	2.0	—	—	2.3	—	2.65	—	—
BV (on rats)	94	84	74	76	65	73	—	—	73	—	—	—	—
NPU	94	82	67	79	40	70	—	—	61	—	—	—	—

[a] Primary limiting acid.
[b] Second limiting acid.
PER, protein efficiency ratio; BV, biological value; NPU, net protein utilization.

Source: Eggum, B. O. and R. M. Beames. 1983. In *Seed Proteins* (Gottschalk, W. and H. P. Muller, Eds.), Nijhoff/Junk, The Hague, pp. 499–531 and FAO/WHO/UNU. 1985. Energy and protein requirements, Report of a joint FAO/WHO/UNU Expert Consultation. World Health Organization Technical Rep. Ser. 724, WHO, Geneva.

TABLE 5.22
Recommended Essential Amino Acid Pattern for Food Proteins

	Recommended Pattern (mg/g Protein)			
Amino Acid	**Infant**	**Preschool Child (2–5 years)**	**Preschool Child (10–12 years)**	**Adult**
Histidine	26	19	19	16
Isoleucine	46	28	28	13
Leucine	93	66	44	19
Lysine	66	58	44	16
Met + Cys	42	25	22	17
Phe + Tyr	72	63	22	19
Threonine	43	34	28	9
Tryptophan	17	11	9	5
Valine	55	35	25	13
Total	434	320	222	111

Source: From FAO/WHO/UNU. 1985. Energy and protein requirements, Report of a joint FAO/WHO/UNU Expert Consultation. World Health Organization Technical Rep. Ser. 724, WHO, Geneva.

TABLE 5.23
Digestibility of Various Food Proteins in Humans

Protein Source	**Digestibility (%)**	**Protein Source**	**Digestibility (%)**
Egg	97	Millet	79
Milk, cheese	95	Peas	88
Meat, fish	94	Peanut	94
Maize	85	Soy flour	86
Rice (polished)	88	Soy protein isolate	95
Wheat, whole	86	Beans	78
Wheat flour, white	96	Corn, cereal	70
Wheat gluten	99	Wheat, cereal	77
Oatmeal	86	Rice cereal	75

Source: From FAO/WHO/UNU. 1985. Energy and protein requirements, Report of a joint FAO/WHO/UNU Expert Consultation. World Health Organization Technical Rep. Ser. 724, WHO, Geneva.

5.7.2.2 Antinutritional Factors

Most plant protein isolates and concentrates contain trypsin and chymotrypsin inhibitors (Kunitz type and Bowman-Birk type) and lectins. These inhibitors impair complete hydrolysis of legume and oilseed proteins by pancreatic proteases. Lectins, which are glycoproteins, bind to intestinal mucosa cells and interfere with absorption of amino acids. Lectins and Kunitz-type protease inhibitors are thermolabile, whereas the Bowman-Birk type inhibitor is stable under normal thermal processing conditions. Thus, heat-treated legume and oilseed proteins are generally more digestible than native protein isolates (despite some residual Bowman-Birk type inhibitor). Plant proteins also contain other antinutritional factors, such as tannins and phytate. Tannins, which are condensation products of polyphenols, covalently react with ε-amino groups of lysine residues. This inhibits trypsin-catalyzed cleavage of the polypeptides at lysine sites.

5.7.2.3 Processing

Interaction of proteins with polysaccharides and dietary fiber also reduces the rate and completeness of hydrolysis. This is particularly important in extruded food products where high temperature and pressure is often used. Proteins undergo several chemical alterations involving lysine residues when exposed to high temperatures and alkaline pH. Such alterations reduce their digestibility. Reaction of reducing sugars with ε-amino groups also decreases digestibility of lysine.

5.7.3 Evaluation of Protein Nutritive Value

Since the nutritional quality of proteins can vary greatly and is affected by many factors, it is important to have procedures for evaluating quality. Quality estimates are useful for: (a) determining the amount required to provide a safe level of essential amino acids for growth and maintenance and (b) monitoring changes in the nutritive value of proteins during food processing, so that processing conditions that minimize quality loss can be devised. The nutritive quality of proteins can be evaluated by several biological, chemical, and enzymatic methods.

5.7.3.1 Biological Methods

Biological methods are based on weight gain or nitrogen retention in test animals when fed with a protein-containing diet. A protein-free diet is used as the control. The protocol recommended by FAO/WHO [39] is generally used for evaluating protein quality. Rats are the usual test animals, although humans are sometimes used. A diet containing about 10% protein on a dry weight basis is used to ensure that the protein intake is below daily requirements. Adequate energy is supplied in the diet. Under these conditions, protein in the diet is utilized to the maximum possible extent for growth. The number of test animals used must be sufficient to assure results that are statistically reliable. A test period of 9 days is common. During each day of the test period, the amount (g) of diet consumed is tabulated for each animal, and the feces and urine are collected for nitrogen analysis.

The data from animal feeding studies are used in several ways to evaluate protein quality. The *protein efficiency ratio* (PER) is the weight (in grams) gained per gram protein consumed. This is a simple and commonly used expression. Another useful expression is *net protein ratio* (NPR). This is calculated as follows:

$$\text{NPR} = \frac{(\text{weight gain}) - (\text{weight loss of protein} - \text{free group})}{\text{protein ingested}} \tag{5.75}$$

NPR values provide information on the ability of proteins to support both maintenance and growth. Since rats grow much faster than humans, and a larger percentage of protein is used for maintenance in growing children than in rats, it is often questioned whether PER and NPR values derived from rat studies are useful for estimating human needs [108]. Although this argument is a valid one, appropriate correction procedures are available.

Another approach to evaluating protein quality involves measuring nitrogen uptake and nitrogen loss. This allows calculation of two useful protein quality parameters. *Apparent protein digestibility* or *coefficient of protein digestibility* is obtained from the difference between the amount of nitrogen ingested and the amount of nitrogen excreted in the feces. However, since total fecal nitrogen also includes metabolic or endogenous nitrogen, correction should be made to obtain *true protein digestibility*. True digestibility (TD) can be calculated in the following manner:

$$\text{TD} = \frac{I - (F_{\text{N}} - F_{\text{k,N}})}{I} \times 100 \tag{5.76}$$

where I is nitrogen ingested, F_{N} is total fecal nitrogen, and $F_{\text{k,N}}$ is endogenous fecal nitrogen. $F_{\text{k,N}}$ is obtained by feeding a protein-free diet.

True digestibility gives information on the percentage of nitrogen intake absorbed by the body. However, it does not provide information on how much of the absorbed nitrogen is actually retained or utilized by the body.

Biological value, BV, is calculated as follows:

$$\text{BV} = \frac{I - (F_{\text{N}} - F_{\text{k,N}}) - (U_{\text{N}} - U_{\text{k,N}})}{I - (F_{\text{N}} - F_{\text{k,N}})} \times 100 \tag{5.77}$$

where U_{N} and $U_{\text{k,N}}$ are the total and endogenous nitrogen losses, respectively, in the urine.

Net protein utilization (NPU), that is, the percentage of nitrogen intake retained as body nitrogen, is obtained from the product of TD and BV. Thus,

$$\text{NPU} = \text{TD} \times \text{BV} = \frac{I - (F_{\text{N}} - F_{\text{k,N}}) - (U_{\text{N}} - U_{\text{k,N}})}{I} \times 100 \tag{5.78}$$

The PER, BVs, and NPUs of several food proteins are presented in Table 5.21.

Other bioassays that are occasionally used to evaluate protein quality include assays for: enzyme activity, changes in the essential amino acid content of plasma, levels of urea in the plasma and urine, and rate of repletion of plasma proteins or gain in body weight of animals previously fed a protein-free diet.

5.7.3.2 Chemical Methods

Biological methods are expensive and time consuming. Determining its content of amino acids and comparing this with the essential amino acid pattern of an ideal reference protein can obtain quick assessment of a protein's nutritive value. The ideal pattern of essential amino acids in proteins (reference protein) for preschool children (2–5 years) is given in Table 5.22 [40] and this pattern is used as the standard for all age groups except infants. Each essential amino acid in a test protein is given a *chemical score*, which is defined as

$$\frac{\text{mg amino acid per g test protein}}{\text{mg same amino acid per g reference protein}} \times 100 \tag{5.79}$$

The essential amino acid that shows the lowest score is the most limiting amino acid in the test protein. The chemical score of this limiting amino acid provides the chemical score for the test protein. As mentioned earlier, Lys, Thr, Trp, and sulfur amino acids are often the limiting amino acids in food proteins. Therefore, the chemical scores of these amino acids are often sufficient to evaluate the nutritive value of proteins. The chemical score enables estimation of the amount of a test protein or protein mix needed to meet the daily requirement of the limiting amino acid. This can be calculated as follows:

$$\text{Required intake of protein} = \frac{\text{Recommended intake of egg or milk protein}}{\text{Chemical score of protein}} \times 100 \tag{5.80}$$

One of the advantages of the chemical score method is that it is simple and allows one to determine the complementary effects of proteins in a diet. This also allows one to develop high quality protein diets by mixing various proteins suitable for various feeding programs. There are, however, several drawbacks to use the chemical score method. An assumption underlying chemical score is that all test proteins are fully or equally digestible and that all essential amino acids are fully absorbed. Because this assumption is often violated, correlation between results from bioassays and chemical scores is often not good. However, the correlation improves when chemical scores are corrected for protein digestibility. The apparent digestibility of proteins can be rapidly determined *in vitro* using

a combination of three or four enzymes, such as trypsin, chymotrypsin, peptidase, and bacterial protease.

Another shortcoming of chemical score is that it does not distinguish between D- and L-amino acids. Since only L-amino acids are usable in animals, the chemical score overestimates the nutritive value of a protein, especially in proteins exposed to high pH, which cause racemization. The chemical score method is also incapable of predicting the negative effects of high concentrations of one essential amino acid on the bioavailability of other essential amino acids, and it also does not account for the effect of antinutritional factors, such as protease inhibitors and lectins, that might be present in the diet. Despite these major drawbacks, recent findings indicate that chemical scores when corrected for protein digestibility correlate well with biological assays for those proteins having BVs above 40%; when the BV is below 40%, the correlation is poor [39].

5.7.3.3 Enzymic and Microbial Methods

In vitro enzymic methods are sometimes used to measure the digestibility and release of essential amino acids. In one method, test proteins are first digested with pepsin and then with pancreatin (freeze-dried powder of pancreatic extract) [83]. In another method, proteins are digested with three enzymes, namely, pancreatic trypsin, chymotrypsin, and porcine intestinal peptidase, under standard assay conditions [39]. These methods, in addition to providing information on innate digestibility of proteins, are useful for detecting processing-induced changes in protein quality.

Growth of several microorganisms, such as *Streptococcus zymogenes*, *Streptococcus faecalis*, *Leuconostoc mesenteroides*, *Clostridium perfringens*, and *Tetrahymena pyriformis* (a protozoan) also have been used to determine the nutritional value of proteins [44]. Of these microorganisms, *T. pyriformis* is particularly useful, because its amino acid requirements are similar to those of rats and humans.

5.8 PROCESSING-INDUCED PHYSICAL, CHEMICAL, AND NUTRITIONAL CHANGES IN PROTEINS

Commercial processing of foods can involve heating, cooling, drying, application of chemicals, fermentation, irradiation, or various other treatments. Of these, heating is most common. This is commonly done to inactivate microorganisms, to inactivate endogenous enzymes that cause oxidative and hydrolytic changes in foods during storage, and to transform an unappealing blend of raw food ingredients into a wholesome and organoleptically appealing food. In addition, proteins such as bovine β-lactoglobulin, α-lactalbumin and soy protein, that sometimes cause allergenic or hypersensitive responses, can sometimes be rendered innocuous by thermal denaturation. Unfortunately, the beneficial effects achieved by heating proteinaceous foods are generally accompanied by changes that can adversely affect the nutritive value and functional properties of proteins. In this section, both desirable and undesirable effects of food processing on proteins will be discussed.

5.8.1 Changes in Nutritional Quality and Formation of Toxic Compounds

5.8.1.1 Effect of Moderate Heat Treatments

Most food proteins are denatured when exposed to moderate heat treatments (60–90°C, 1 h or less). Extensive denaturation of proteins often results in insolublization, which may impair those functional properties that are dependent on solubility. From a nutritional standpoint, partial denaturation of proteins often improves the digestibility and biological availability of essential amino acids.

Several purified plant proteins and egg protein preparations, even though free of protease inhibitors, exhibit poor *in vitro* and *in vivo* digestibility. Moderate heating improves their digestibility without developing toxic derivatives.

In addition to improving digestibility, moderate heat treatment also inactivates several enzymes, such as proteases, lipases, lipoxygenases, amylases, polyphenoloxidase, and other oxidative and hydrolytic enzymes. Failure to inactivate these enzymes can result in development of off-flavors, rancidity, textural changes, and discoloration of foods during storage. For instance, oilseeds and legumes are rich in lipoxygenase. During crushing or cracking of these beans for extraction of oil or protein isolates, this enzyme, in the presence of molecular oxygen, catalyzes oxidation of polyunsaturated fatty acids to initially yield hydroperoxides. These hydroperoxides subsequently decompose and liberate aldehydes and ketones, which impart off-flavor to soy flour and soy protein isolates and concentrates. To avoid off-flavor formation, it is necessary to thermally inactivate lipoxygenase prior to crushing.

Moderate heat treatment is particularly beneficial for plant proteins, because they usually contain proteinaceous antinutritional factors. Legume and oilseed proteins contain several trypsin and chymotrypsin inhibitors. These inhibitors impair efficient digestion of proteins, and thus reduce their biological availability. Furthermore, inactivation and complexation of trypsin and chymotrypsin by these inhibitors induces over production and secretion of these enzymes by the pancreas, which can lead to pancreatic hypertropy (enlargement of the pancreas) and pancreatic adenoma. Legume and oilseed proteins also contain lectins, which are glycoproteins. These are also known as phytohemagglutinins because they cause agglutination of red blood cells. Lectins exhibit a high binding affinity for carbohydrates. When consumed by humans, lectins impair protein digestion [103] and cause intestinal malabsorption of other nutrients. The latter consequence results from binding of lectins to membrane glycoproteins of intestinal mucosa cells, which alters their morphology and transport properties [96]. Both protease inhibitors and lectins found in plant proteins are thermolabile. Toasting of legumes and oilseeds or moist heat treatment of soy flour inactivates both lectins and protease inhibitors, improves the digestibility and PER of these proteins (Figure 5.33) [45], and prevents pancreatic hypertrophy [53]. These antinutritional factors do not pose problems in home-cooked or

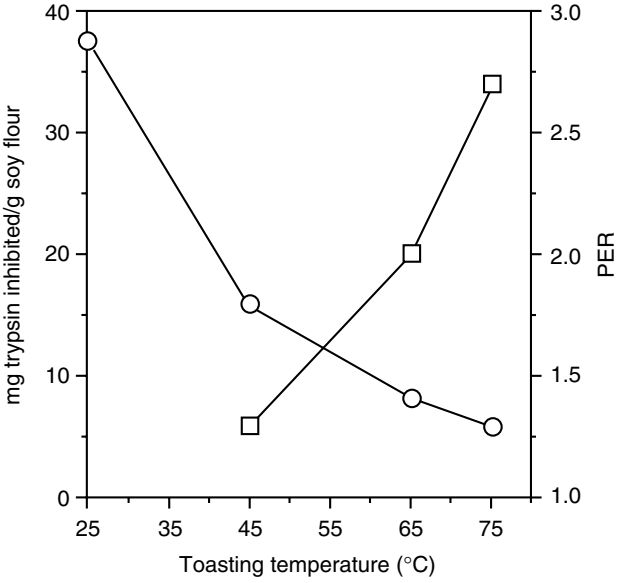


FIGURE 5.33 Effect of toasting on trypsin inhibitory activity and PER of soy flour. (Adapted from Friedman, M. and M. R. Gumbmann. 1986. *Adv. Exp. Med. Biol.* **199**:357–390.)

industrially processed legumes and flour-based products when heating conditions are adequate to inactivate them.

Milk and egg proteins also contain several protease inhibitors. Ovomucoid, which possesses antitryptic activity, constitutes about 11% of egg albumen. Ovoinhibitor, which inhibits trypsin, chymotrypsin, and some fungal proteases, is present at a 0.1% level in egg albumen. Milk contains several protease inhibitors, such as plasminogen activator inhibitor (PAI) and plasmin inhibitor (PI), derived from blood. All of these inhibitors lose their activity when subjected to moderate heat treatment in the presence of water.

The beneficial effects of heat treatment also include inactivation of protein toxins, such as botulinum toxin from *Clostridium botulinum* (inactivated by heating at 100°C) and enterotoxin from *Staphylococcus aureus*.

5.8.1.2 Compositional Changes During Extraction and Fractionation

Preparation of protein isolates from biological sources involves several unit operations, such as extraction, isoelectric precipitation, salt precipitation, thermocoagulation, and ultrafiltration/diafiltration. It is very likely that some of the proteins in the crude extract might be lost during some of these operations. For example, during isoelectric precipitation, some sulfur-rich albumin-type proteins, which are usually soluble at isoelectric pH, might be lost in the supernatant fluid. Such losses can alter the amino acid composition and nutritional value of protein isolates compared to those of crude extracts. For instance, WPC prepared by ultrafiltration/diafiltration and ion exchange methods undergo marked changes in their proteose–peptone contents. This markedly affects their foaming properties.

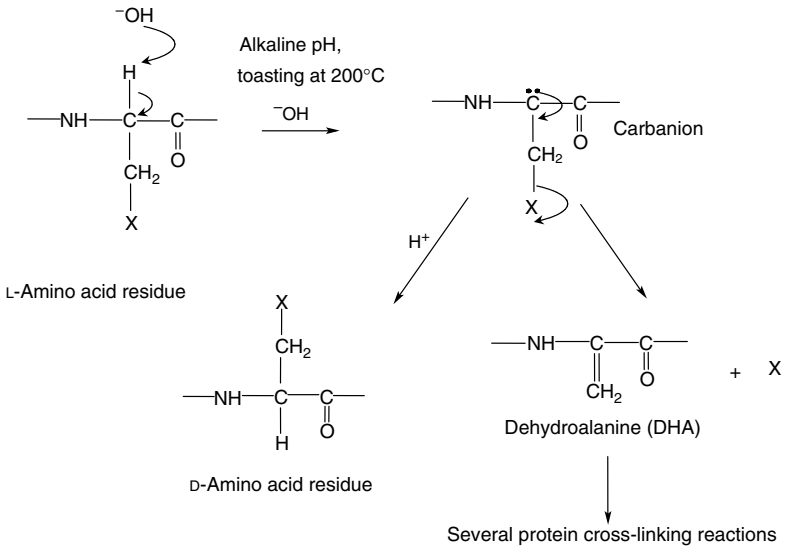
5.8.1.3 Chemical Alteration of Amino Acids

Proteins undergo several chemical changes when processed at high temperatures. These changes include racemization, hydrolysis, desulfuration, and deamidation. Most of these chemical changes are irreversible, and some of these reactions result in formation of modified amino acid types that are potentially toxic.

5.8.1.3.1 Racemization

Thermal processing of proteins at alkaline pH, as is done to prepare texturized foods, invariably leads to partial racemization of L-amino acid residues to D-amino acids [77]. Acid hydrolysis of proteins also causes some racemization of amino acids [42] as does roasting of proteins or protein containing foods above 200°C [55]. The mechanism at alkaline pH involves initial abstraction of the proton from the α-carbon atom by a hydroxyl ion. The resulting carbanion loses its tetrahedral asymmetry. Subsequent addition of a proton from solution can occur either from the top or bottom of the carbanion. This equal probability results in racemization of the amino acid residue (Equation 5.81) [77]. The rate of racemization of a residue is affected by the electron withdrawing power of the side chain. Thus, residues such as Asp, Ser, Cys, Glu, Phe, Asn, and Thr are racemized at a faster rate than are other amino acid residues [78]. The rate of racemization is also dependent on hydroxyl ion concentration, but is independent of protein concentration. Interestingly, the rate of racemization is about ten times faster in proteins than in free amino acids [78], suggesting that intramolecular forces in a protein reduce the activation energy of racemization. In addition to racemization, the carbanion formed under alkaline pH also can undergo β-elimination reaction to yield a reactive intermediate dehydroalanine. Cysteine and phosphoserine residues display greater propensity for this route than other amino acid residues. This is one of the reasons why a significant amount of D-cysteine is not

found in alkali treated proteins.

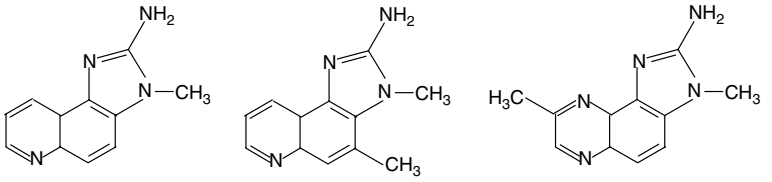


(5.81)

Racemization of amino acid residues causes a reduction in protein digestibility because the peptide bonds involving D-amino acid residues are less efficiently hydrolyzed by gastric and pancreatic proteases. This leads to loss of essential amino acids that have racemized, and impairs the nutritional value of the protein. D-Amino acids are also less efficiently absorbed through intestinal mucosa cells and even if absorbed, they cannot be utilized in *in vivo* protein synthesis. Moreover, some D-amino acids, for example, D-proline, have been found to be neurotoxic in chickens [20].

In addition to racemization and β-elimination reactions, heating of proteins at alkaline pH destroys several amino acid residues, such as Arg, Ser, Thr, and Lys. Arg decomposes to ornithine.

When proteins are heated above 200°C, as is commonly encountered on food surfaces during broiling, baking, and grilling, amino acid residues undergo decomposition and pyrolysis. Several of the pyrolysis products have been isolated and identified from broiled and grilled meat, and they are highly mutagenic as determined by the Ames test. The most carcinogenic/mutagenic products are formed from pyrolysis of Trp and Glu residues [19]. Pyrolysis of Trp residues gives rise to formation of carbolines and their derivatives. Mutagenic compounds are also produced in meats at moderate temperatures (190–200°C). These are known as IQ (imidazo quinolines) compounds, which are condensation products of creatine, sugars, and certain amino acids, such as Gly, Thr, Ala, and Lys [60]. The three most potent mutagens formed in broiled fish are shown below.



2-Amino-3-methylimidazo-[4,5-*f*]quinoline (IQ)

2-Amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ)

2-Amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx)

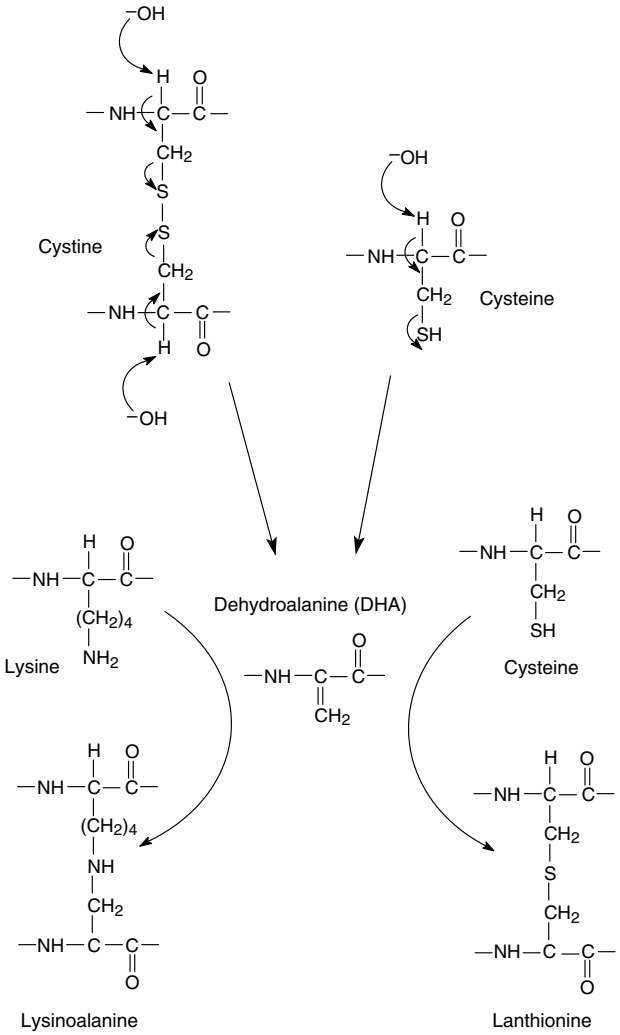
(5.82)

Following heating of foods according to recommended procedures, IQ compounds are generally present only at very low concentrations (μg amounts).

5.8.1.3.2 Protein Cross-linking

Several food proteins contain both intra- and intermolecular cross-links, such as disulfide bonds in globular proteins, desmosine, and isodesmosine, and di- and trityrosine type cross-links in fibrous proteins such as keratin, elastin, resilin, and collagen. Collagen also contains ε-*N*-(γ-glutamyl)lysyl and/or ε-*N*-(γ-aspartyl)lysyl cross-links. One of the functions of these cross-links in native proteins is to minimize proteolysis *in vivo*. Processing of food proteins, especially at alkaline pH, also induces cross-link formation. Such unnatural covalent bonds between polypeptide chains reduce digestibility and biological availability of essential amino acids that are involved in, or near, the cross-link.

As discussed in the previous section, heating at alkaline pH or heating above 200°C at neutral pH, results in abstraction of the proton from the α-carbon atom resulting in formation of a carbanion, which leads to formation of dehydroalanine (DHA) residue. DHA formation can also occur via a one-step mechanism without the carbanion intermediate. Once formed, the highly reactive DHA residues react with nucleophilic groups, such as the ε-amino group of a lysyl residue, the thiol group of Cys residue, the δ-amino group of ornithine (formed by decomposition of arginine), or a histidyl residue, resulting in formation of lysinoalanine, lanthionine, ornithoalanine, and histidinylalanine cross-links, respectively, in proteins. Lysinoalanine is the major cross-link commonly found in alkali treated proteins because of the abundance of readily accessible lysyl residues (Equation 5.83).



(5.83)

The formation of protein–protein cross-links in alkali-treated proteins decreases their digestibility and biological value. The decrease in digestibility is related to the inability of trypsin to cleave the peptide bond in the lysinoalanine cross-link. Moreover, the steric constraints imposed by the cross-links also prevent hydrolysis of other peptide bonds in the neighborhood of the lysinoalanine and similar cross-links. Evidence suggests that free lysinoalanine is absorbed in the intestine, but the body does not utilize it and most of it is excreted in the urine. Some lysinoalanine is metabolized in the kidney. The inability of the body to cleave the lysinoalanine covalent bond reduces the bioavailability of lysine in alkali-treated proteins.

Rats fed 100 ppm of pure lysinoalanine or 3000 ppm of protein-bound lysinoalanine develop nephrocytomegaly (i.e., kidney disorder). However, such nephrotoxic effects have not been observed in other animal species, such as quails, mice, hamsters, and monkeys. This has been attributed to differences in the types of metabolites formed in rats vs other animals. At levels encountered in foods, protein-bound lysinoalanine apparently does not cause nephrotoxicity in humans. Nevertheless, minimization of lysinoalanine formation during alkali processing of proteins is a desirable goal.

The lysinoalanine contents of several commercial foods are listed in Table 5.24 [120]. The extent of formation of lysinoalanine is dependent on pH and temperature. The higher the pH, the greater is the extent of lysinoalanine formation. High temperature heat treatment of foods, such as milk, causes a significant amount of lysinoalanine to form even at neutral pH. Lysinoalanine formation in proteins can be minimized or inhibited by adding small molecular-weight nucleophilic compounds, such as cysteine, ammonia, or sulfites. The effectiveness of cysteine results because the nucleophilic

TABLE 5.24
Lysinoalanine (LAL) Content of Processed Foods

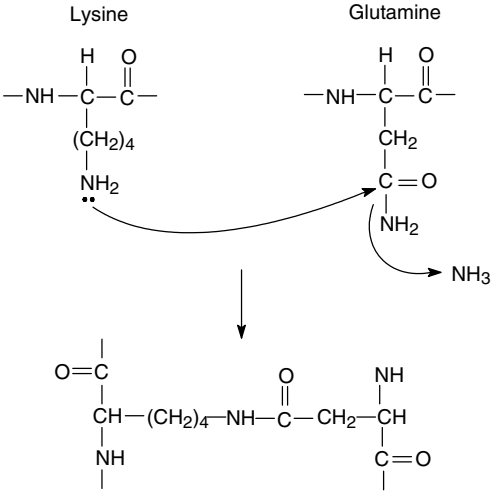
Food	LAL (μg/g Protein)
Corn chips	390
Pretzels	500
Hominy	560
Tortillas	200
Taco shells	170
Milk, infant formula	150–640
Milk, evaporated	590–860
Milk, condensed	360–540
Milk, UHT	160–370
Milk, HTST	260–1,030
Milk, spray-dried powder	0
Skim milk, evaporated	520
Simulated cheese	1,070
Egg white solids, dried	160–1,820
Calcium caseinate	370–1,000
Sodium caseinate	430–6,900
Acid casein	70–190
Hydrolyzed vegetable protein	40–500
Whipping agent	6,500–50,000
Soy protein isolate	0–370
Yeast extract	120

Source: Swaisgood, H. E. and G. L. Catignani. 1991. *Adv. Food Nutr. Res*. **35**:185–236.

SH group reacts more than 1000 times faster than the ε-amino group of lysine. Sodium sulfite and ammonia exert their inhibitory effect by competing with the ε-amino group of lysine for DHA. Blocking of ε-amino groups of lysine residues by reaction with acid anhydrides prior to alkali-treatment also decreases the formation of lysinoalanine. However, this approach results in loss of lysine and may be unsuitable for food applications.

Under normal conditions used for processing of several foods, only small amounts of lysinoalanine are formed. Thus, toxicity of lysinoalanine in alkali-treated foods is not believed to be a major concern. However, reduction in digestibility, loss of bioavailability of lysine, and racemization of amino acids (some of which are toxic) are all undesirable outcomes in alkali-treated foods such as texturized vegetable proteins.

Excessive heating of pure protein solutions or proteinaceous foods low in carbohydrate content also results in formation of ε-*N*-(γ-glutamyl)lysyl and ε-*N*-(γ-aspartyl)lysyl cross-links. These involve a transamidation reaction between Lys and Gln or Asn residues (Equation 5.84). The resulting cross-links are termed isopeptide bonds because they are foreign to native proteins. Isopeptides resist enzymatic hydrolysis in the gut and these cross-linkages therefore impair digestibility of proteins, and bioavailability of lysine.



(5.84)

ε-*N*-(γ-Glutamyl)lysine cross-link

Ionizing radiation of foods results in the formation of hydrogen peroxide through radiolysis of water in the presence of oxygen, and this, in turn, causes oxidative changes in, and polymerization of, proteins. Ionizing radiation also may directly produce free radicals via ionization of water.

$$H_2O \rightarrow H_2O^+ + e^- \tag{5.85}$$

$$H_2O^+ + H_2O \rightarrow H_3O^+ + {}^{\bullet}OH \tag{5.86}$$

The hydroxyl free radical can induce formation of protein free radicals, which in turn may cause polymerization of proteins.

$$P + {}^{\bullet}OH \rightarrow P^{\bullet} + H_2O \tag{5.87}$$

$$\begin{aligned} P^{\bullet} + P^{\bullet} &\rightarrow P - P \\ P^{\bullet} + P &\rightarrow P - P^{\bullet} \end{aligned} \tag{5.88}$$

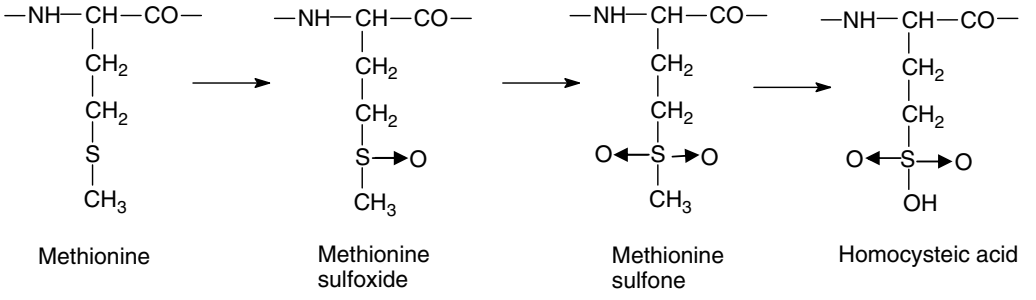
Heating of protein solutions at 70–90°C and at neutral pH generally leads to sulfhydryl–disulfide interchange reactions (if these groups are present), resulting in polymerization of proteins. However, this type of heat-induced cross-link generally does not have an adverse effect on the digestibility of proteins and bioavailability of essential amino acids because these bonds can be broken *in vivo*.

5.8.1.4 Effects of Oxidizing Agents

Oxidizing agents such as hydrogen peroxide and benzoyl peroxide are used as bactericidal agents in milk, as bleaching agents in cereal flours, protein isolates, and fish protein concentrate, and for detoxification of oilseed meals. Sodium hypochlorite is also commonly used as a bactericidal and detoxifying agent in flours and meals. In addition to oxidizing agents that are sometimes added to foods, several oxidative compounds are endogenously produced in foods during processing. These include free radicals formed during irradiation of foods, during peroxidation of lipids, during photooxidation of compounds such as riboflavin and chlorophyll, and during nonenzymatic browning of foods. In addition, polyphenols present in several plant protein isolates can be oxidized by molecular oxygen to quinones at neutral to alkaline pH, and this will lead ultimately to peroxides. These highly reactive oxidizing agents cause oxidation of several amino acid residues and polymerization of proteins. The amino acid residues most susceptible to oxidation are Met, Cys, Trp, and His, and to a lesser extent Tyr.

5.8.1.4.1 Oxidation of Methionine

Methionine is easily oxidized to methionine sulfoxide by various peroxides. Incubation of protein bound methionine or free methionine with hydrogen peroxide (0.1 M) at elevated temperature for 30 min results in complete conversion of methionine to methionine sulfoxide [23]. Under strong oxidizing conditions, methionine sulfoxide is further oxidized to methionine sulfone, and in some cases to homocysteic acid.

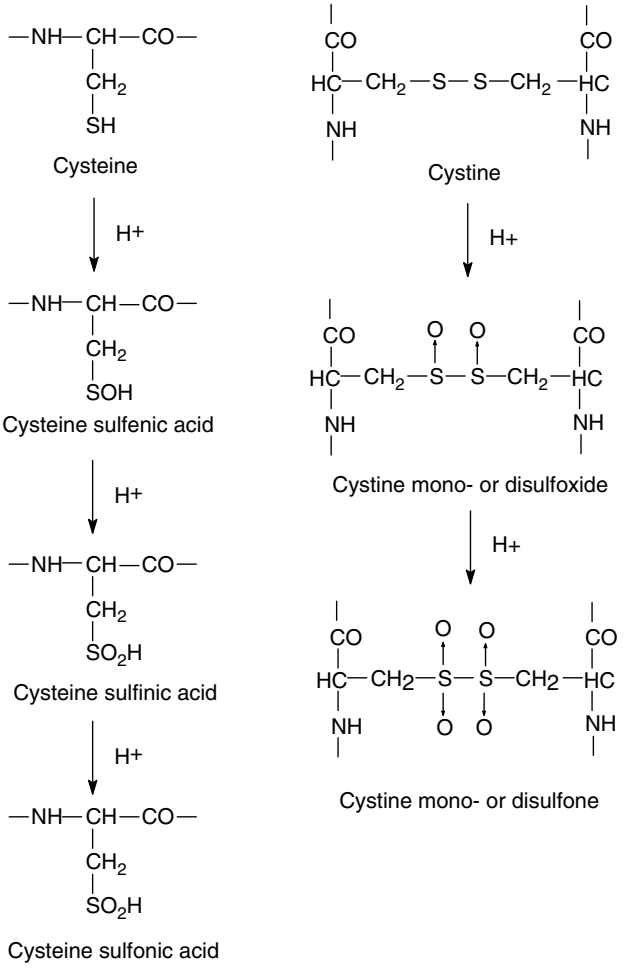


(5.89)

Methionine becomes biologically unavailable once it is oxidized to methionine sulfone or homocysteic acid. Methionine sulfoxide, on the other hand, is reconverted to Met under acidic conditions in the stomach. Further, evidence suggests that any methionine sulfoxide passing through the intestine is absorbed and reduced *in vivo* to methionine. However, *in vivo* reduction of methionine sulfoxide to methionine is slow. The PER or NPU of casein oxidized with 0.1 M hydrogen peroxide (which completely transforms methionine to methionine sulfoxide) is about 10% less than that of control casein.

5.8.1.4.2 Oxidation of Cysteine and Cystine

Under alkaline conditions, cysteine and cystine follow the β-elimination reaction pathway to produce DHA. However, at acidic pH, oxidation of cysteine and cystine in simple systems results in formation of several intermediate oxidation products. Some of these derivatives are unstable.



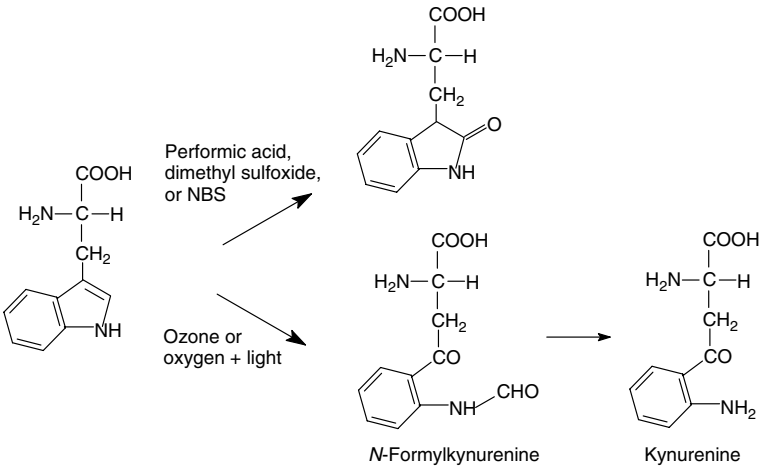
(5.90)

Mono- and disulfoxides of L-cystine are biologically available, presumably because they are reduced back to L-cystine in the body. However, mono- and disulfone derivatives of L-cystine are biologically unavailable. Similarly, while cysteine sulfenic acid is biologically available, cysteine sulfinic acid and cysteic acid are not. The rate and extent of formation of these oxidation products in acidic foods are not well documented.

5.8.1.4.3 Oxidation of Tryptophan

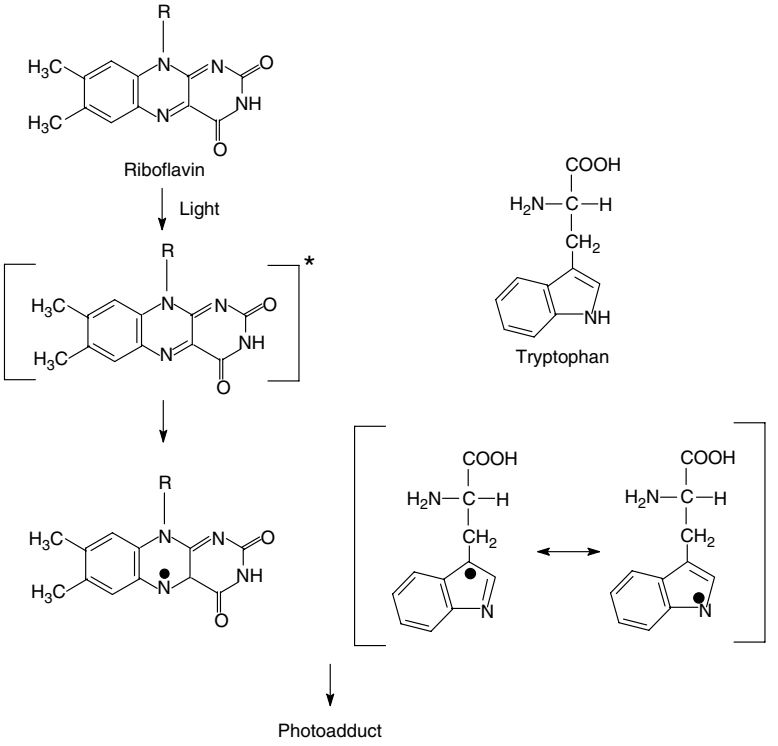
Among the essential amino acids, Trp is exceptional because of its role in several biological functions. Therefore, its stability in processed foods is of major concern. Under acidic, mild, oxidizing conditions, such as in the presence of performic acid, dimethylsulfoxide, or *N*-bromosuccinimide (NBS), Trp is oxidized mainly to β-oxyindolylalanine. Under acidic, severe, oxidizing conditions, such as in the presence of ozone, hydrogen peroxide or peroxidizing lipids, Trp is oxidized to

N-formylkynurenine, kynurenine, and other unidentified products.



(5.91)

Exposure of Trp to light in the presence of oxygen and a photosensitizer, such as riboflavin or chlorophyll, leads to formation of *N*-formylkynurenine and kynurenine as major products and several other minor ones. Depending upon the pH of the solution, other derivatives, such as 5-hydroxyformylkynurenine (pH > 7.0) and a tricyclic hydroperoxide (pH 3.6–7.1), are also formed [86]. In addition to the photooxidative products, Trp forms a photoadduct with riboflavin.



(5.92)

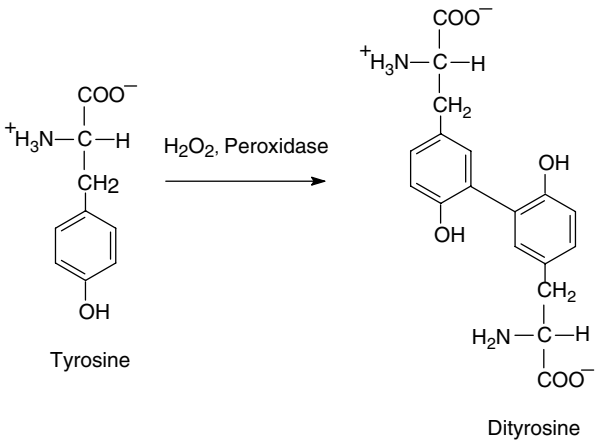
Both protein-bound and free tryptophan is capable of forming this adduct. The extent of formation of this photoadduct is dependent on availability of oxygen, being greater under anaerobic conditions [112].

The oxidation products of Trp are biologically active. In addition, kynurenines are carcinogenic in animals, and all other Trp photo-oxidation products as well as the carbolines formed during broiling/grilling of meat products exhibit mutagenic activities and inhibit growth of mammalian cells in tissue cultures. The tryptophan–riboflavin photoadduct shows cytotoxic effects on mammalian cells, and exerts hepatic dysfunctions during parenteral nutrition. These undesirable products are normally present in extremely low concentration in foods unless an oxidation environment is purposely created.

Among the amino acid side chains, only those of Cys, His, Met, Trp, and Tyr are susceptible to sensitized-photooxidation. In the case of Cys, cysteic acid is the end product. Met is photooxidized first to methionine sulfoxide, and finally to methionine sulfone and homocysteic acid. Photooxidation of histidine leads to the formation of aspartate and urea. The photooxidation products of tyrosine are not known. Since foods contain endogenous as well as supplemented riboflavin (vitamin B2), and usually are exposed to light and air, some degree of sensitized-photooxidation of the above amino acid residues would be expected to occur. In milk, free methionine is converted to methional by light-activated oxidation, which imparts a characteristic flavor to the milk. At equimolar concentrations, the rates of oxidation of the sulfur amino acids and Trp are likely to follow the order Met > Cys > Trp.

5.8.1.4.4 Oxidation of Tyrosine

Exposure of tyrosine solutions to peroxidase and hydrogen peroxide results in oxidation of tyrosine to dityrosine. Occurrence of this type of cross-link has been found in natural proteins, such as resilin, elastin, keratin, and collagen, and more recently in doughs.



(5.93)

5.8.1.5 Carbonyl–Amine Reactions

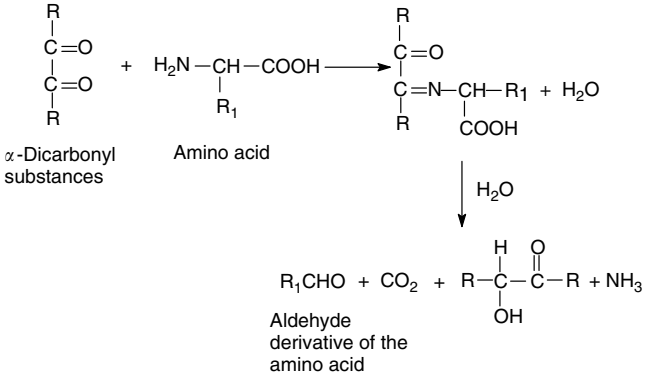
Among the various processing-induced chemical changes in proteins, the Maillard reaction (nonenzymatic browning) has the greatest impact on its sensory and nutritional properties. The Maillard reaction refers to a complex set of reactions initiated by reaction between amines and carbonyl compounds, which, at elevated temperature, decompose and eventually condense into insoluble brown product known as melanoidins (see Chapter 14). This reaction occurs not only in foods during processing, but also in biological systems. In both instances, proteins and amino acids typically provide the amino component, and reducing sugars (aldoses and ketoses), ascorbic acid, and carbonyl compounds generated from lipid oxidation provide the carbonyl component.

Some of the carbonyl derivatives from the nonenzymatic browning sequence react readily with free amino acids. This results in degradation of the amino acids to aldehydes, ammonia, and carbon dioxide and the reaction is known as *Strecker degradation.* The aldehydes contribute to aroma development during the browning reaction. Strecker degradation of each amino acid produces a

TABLE 5.25
Characteristic Flavor Notes of Aldehydes Produced by Strecker Degradation of Amino Acids

Amino Acid	Typical Flavor
Phe, Gly	Caramel-like
Leu, Arg, His	Bread-like, toasted
Ala	Nutty
Pro	Bakery, cracker
Gln, Lys	Buttery
Met	Broth, beany
Cys, Gly	Smokey, burnt
α-Amino butyric acid	Walnut
Arg	Popcorn-like

specific aldehyde with a distinctive aroma (Table 5.25).



(5.94)

The Maillard reaction impairs protein nutritional value. Some of the products are antioxidants and some may be toxic; but the toxic products probably are not hazardous at concentrations encountered in foods. Because the ε-amino group of lysine is the major source of primary amines in proteins, it is frequently involved in the carbonyl–amine reaction and typically suffers a major loss in bioavailability when this reaction occurs. The extent of Lys loss depends on the stage of the browning reaction. Lysine involved in the early stages of browning, including the Schiff's base, is biologically available. These early derivatives are hydrolyzed to lysine and sugar in the acidic conditions of the stomach. However, beyond the stage of ketosamine (Amadori product) or aldosamine (Heyns product), lysine is no longer biologically available. This is primarily because of poor absorption of these derivatives in the intestine [36]. It is important to note that no color has developed at this stage. Although sulfite inhibits formation of brown pigments [129], it cannot prevent loss of lysine availability, because it cannot prevent formation of Amadori or Heyns products.

Biological activity of lysine at various stages of the Maillard reaction can be determined chemically by addition of 1-fluoro-2,4-dinitrobenzene (FDNB), followed by acid hydrolysis of the derivatized protein. FDNB reacts with available ε-amino groups of lysyl residues. The hydrolysate is then extracted with ethyl ether to remove unreacted FDNB, and the concentration of ε-dinitrophenyl-lysine (ε-DNP-lysine) in the aqueous phase is determined by measuring absorbance at 435 nm. Available lysine also can be determined by reacting 2,4,6-trinitrobenzene sufonic acid (TNBS) with the ε-amino group. In this case, the concentration of ε-trinitrophenyl-lysine (ε-TNP-lysine) derivative is determined from absorbance at 346 nm.

Nonenzymatic browning not only causes major losses of lysine, but reactive unsaturated carbonyls and free radicals formed during the browning reaction cause oxidation of several other essential amino acids, especially Met, Tyr, His, and Trp. Cross-linking of proteins by dicarbonyl compounds produced during browning decreases protein solubility and impairs digestibility of proteins.

Some of Maillard brown products are suspected mutagens. Although mutagenic compounds are not necessarily carcinogenic, all known carcinogens are mutagens. Therefore, the formation of mutagenic Maillard compounds in foods is of concern. Studies with mixtures of glucose and amino acids have shown that the Maillard products of Lys and Cys are mutagenic, whereas those of Trp, Tyr, Asp, Asn, and Glu are not, as determined by the Ames test. It should be pointed out that pyrolysis products of Trp and Glu (in grilled and broiled meat) also are mutagenic (Ames test). As discussed earlier, heating of sugar and amino acids in the presence of creatine produces the most potent IQ-type mutagens (see Equation 5.82). Although results based on model systems cannot be reliably applied to foods, it is possible that interaction of Maillard products with other small molecular-weight constituents in foods may produce mutagenic and/or carcinogenic substances.

On a positive note, some Maillard reaction products, especially the reductones, do have antioxidative activity [88]. This is due to their reducing power and their ability to chelate metals, such as Cu and Fe that are prooxidants. The amino reductones formed from the reaction of triose reductones with amino acids such as Gly, Met, and Val show excellent antioxidative activity.

Besides reducing sugars, other aldehydes and ketones present in foods can also take part in the carbonyl–amine reaction. Notably, gossypol (in cotton seed), glutaraldehyde (added to protein meals to control deamination in the rumen of ruminants), and aldehydes (especially malonaldehyde) generated from the oxidation of lipids may react with amino groups of proteins. Bifunctional aldehydes, such as malonaldehyde can cross-link and polymerize proteins. This may result in insolublization, loss of digestibility and bioavailability of lysine, and loss of functional properties of proteins. Formaldehyde also reacts with the ε-amino group of lysine residues; the toughening of cod-type fish muscle during frozen storage is believed to be due to reactions of formaldehyde with fish proteins.

$$\underset{\text{Protein amino group}}{\text{P}{-}\text{NH}_2} + \underset{\text{Malondialdehyde}}{\text{OHC}{-}\text{CH}_2{-}\text{CHO}} \rightarrow \underset{\text{Protein–Protein cross-linkage}}{\text{P}{-}\text{N}{=}\text{CH}{-}\text{CH}_2{-}\text{CH}{=}\text{N}{-}\text{P}} \tag{5.95}$$

5.8.1.6 Other Reactions of Proteins in Foods

5.8.1.6.1 Reactions with Lipids

Oxidation of unsaturated lipids leads to formation of alkoxy and peroxy free radicals. These free radicals in turn react with proteins, forming lipid–protein free radicals. These lipid–protein conjugated free radicals can undergo polymerization cross-linking of proteins leading to a variety of cross-linked products.

$$\text{LH} + \text{O}_2 \rightarrow \text{LOO}^{\bullet} \tag{5.96}$$

$$\text{LOO}^{*} + \text{LH} \rightarrow \text{LOOH} + \text{L}^{\bullet} \tag{5.97}$$

$$\text{LOOH} \rightarrow \text{LO}^{\bullet} + \text{HO}^{\bullet} \tag{5.98}$$

$$\text{LO}^{\bullet} + \text{PH} \rightarrow \text{LOP} \tag{5.99}$$

$$\text{LOP} + \text{LO}^{\bullet} \rightarrow {}^{\bullet}\text{LOP} + \text{LOH} \tag{5.100}$$

$${}^{\bullet}\text{LOP} + {}^{\bullet}\text{LOP} \rightarrow \text{POLLOP} \tag{5.101}$$

or

$$LOO^{\bullet} + PH \rightarrow LOOP \quad (5.102)$$

$$LOOP + LOO^{\bullet} \rightarrow {}^{\bullet}LOOP + LOOH \quad (5.103)$$

$${}^{\bullet}LOOP + {}^{\bullet}LOOP \rightarrow POOLLOOP \quad (5.104)$$

$${}^{\bullet}LOOP + {}^{\bullet}LOP \rightarrow POLLOOP \quad (5.105)$$

In addition, the lipid free radicals can also induce formation of protein free radicals at cysteine and histidine side chains, which may then undergo cross-linking and polymerization reactions.

$$LOO^{*} + PH \rightarrow LOOH + P^{*} \quad (5.106)$$

$$LO^{\bullet} + PH \rightarrow LOH + P^{\bullet} \quad (5.107)$$

$$P^{\bullet} + P^{\bullet} \rightarrow P - P^{\bullet} \quad (5.108)$$

$$P - P^{\bullet} + PH \rightarrow P - P - P^{\bullet} \quad (5.109)$$

$$P - P - P^{\bullet} + P^{\bullet} \rightarrow P - P - P - P \quad (5.110)$$

Lipid hydroperoxides (LOOH) in foods can decompose, resulting in liberation of aldehydes and ketones, notably malonaldehyde. These carbonyl compounds react with amino groups of proteins via carbonyl–amine reaction and Schiff's base formation. As discussed earlier, reaction of malonaldehye with lysyl side chains leads to cross-linking and polymerization of proteins. The reaction of peroxidizing lipids with proteins generally has deleterious effects on nutritional value of proteins. Noncovalent binding of carbonyl compounds to proteins also imparts off-flavors.

5.8.1.6.2 Reactions with Polyphenols

Phenolic compounds, such as *p*-hydroxybenzoic acid, catechol, caffeic acid, gossypol, and quercein, are found in all plant tissues. During maceration of plant tissues, these phenolic compounds can be oxidized by molecular oxygen at alkaline pH to quinones. This can also occur by the action of polyphenoloxidase, which is commonly present in plant tissues. These highly reactive quinones can irreversibly react with the sulfhydryl and amino groups of proteins. Reaction of quinones with SH and α-amino groups (N-terminal) is much faster than it is with ε-amino groups. In addition, quinones can also undergo condensation reactions, resulting in formation of high molecular weight brown color pigments. These brown products remain highly reactive and readily combine with SH and amino groups of proteins. Quinone–amino group reactions decrease the digestibility and bioavailability of protein-bound lysine and cysteine.

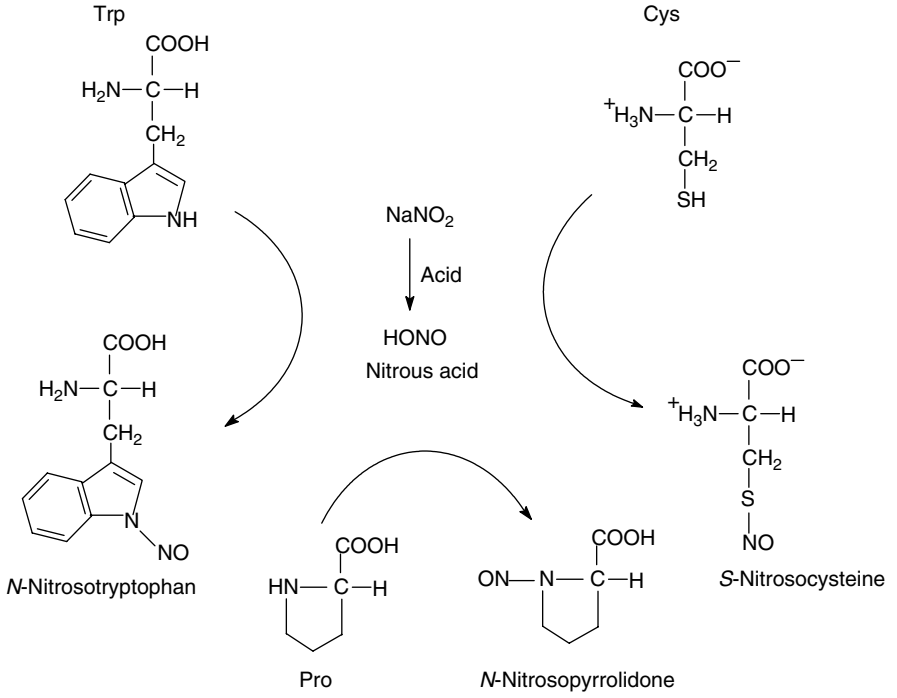
5.8.1.6.3 Reactions with Halogenated Solvents

Halogenated organic solvent are often used to extract oil and some antinutritive factors from oilseed products, such as soybean and cottonseed meals. Extraction with trichloroethylene results in formation of a small amount of *S*-dichlorovinyl-L-cysteine, which is toxic. On the other hand, the solvents dichloromethane and tetrachloroethylene do not seem to react with proteins. 1,2-Dichloroethane reacts with Cys, His, and Met residues in proteins. Certain fumigants, such as methyl bromide, can alkylate Lys, His, Cys, and Met residues. All of these reactions decrease the nutritional value of proteins and some are of concern from a safety standpoint.

5.8.1.6.4 Reactions with Nitrites

Reaction of nitrites with secondary amines, and to some extent with primary and tertiary amines, results in formation of *N*-nitrosoamine, which is one of the most carcinogenic compounds formed in foods. Nitrites are usually added to meat products to improve color and to prevent bacterial growth.

The amino acids (or residues) primarily involved in this reaction are Pro, His, and Trp. Arg, Tyr, and Cys also can react with nitrites. The reaction occurs mainly under acidic conditions and at elevated temperatures.

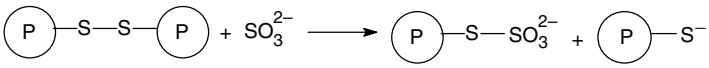


(5.111)

The secondary amines produced during the Maillard reaction, such as Amadori and Heyns products, also can react with nitrites. Formation of *N*-nitrosamines during cooking, grilling, and broiling of meat has been a major concern, but additives, such as ascorbic acid and erythorbate, are effective in curtailing this reaction.

5.8.1.6.5 Reaction with Sulfites

Sulfites reduce disulfide bonds in proteins to yield *S*-sulfonate derivatives. They do not react with cysteine residues.



(5.112)

In the presence of reducing agents, such as cysteine or mecaptoethanol, the *S*-sulfonate derivatives are converted back to cysteine residues. *S*-Sulfonates decompose under acidic (as in stomach) and alkaline pH to disulfides. The *S*-sulfonation does not decrease the bioavailability of cysteine. The increase in electronegativity and the breakage of disulfide bonds in proteins upon *S*-sulfonation causes unfolding of protein molecules, which affects their functional properties.

5.8.2 Changes in the Functional Properties of Proteins

The methods or processes used to isolate proteins can affect their functional properties. Minimum denaturation during various isolation steps is generally desired because this helps to retain acceptable protein solubility, which is often a prerequisite to functionality of these proteins in food products.

In some instances, controlled or partial denaturation of proteins can improve certain functional properties.

Proteins are often isolated using isoelectric precipitation. The secondary, tertiary, and quaternary structures of most globular proteins are stable at their isoelectric pH, and the proteins readily become soluble again when dispersed at neutral pH. On the other hand, protein entities such as casein micelles are irreversibly destabilized by isoelectric precipitation. The collapse of micellar structure in isoelectrically precipitated casein is due to several factors, including solubilization of colloidal calcium phosphate and the change in the balance of hydrophobic and electrostatic interactions among the various casein types. The compositions of isoelectrically precipitated proteins are usually altered from those of the raw materials. This is because some minor proteins fractions are reasonably soluble at the isoelectric pH of the major component and are therefore do not precipitate. This change in composition affects the functional properties of the protein isolate.

Ultrafiltration (UF) is widely used to prepare WPCs. Both protein and nonprotein composition of WPC are affected by removal of small solutes during UF. Partial removal of lactose and ash, strongly influence the functional properties of WPC. Furthermore, increased protein–protein interactions occur in the UF concentrate during exposure to moderate temperatures (50–55°C) and this decreases solubility and stability of the ultrafiltered protein, which in turn changes its water binding capacity and alters its properties with respect to gelation, foaming, and emulsification. Among the ash constituents, variations in calcium and phosphate content significantly affect the gelling properties of WPC. Whey protein isolates prepared by ion exchange contain little ash, and because of this they have functional properties that are superior to those of isolates obtained by ultrafiltration/diafiltration.

Calcium ions often induce aggregation of proteins. This is attributable to formation of ionic bridges involving Ca^{2+} ions and the carboxyl groups. The extent of aggregation depends on calcium ion concentration. Most proteins show maximum aggregation at 40–50 mM Ca^{2+} ion concentration. With some proteins, such as caseins and soy proteins, calcium aggregation leads to precipitation, whereas, in the case of whey protein isolate, a stable colloidal aggregate forms (Figure 5.34).

Exposure of proteins to alkaline pH, particularly at elevated temperatures, causes irreversible conformational changes. This is partly because of deamidation of Asn and Gln residues, and

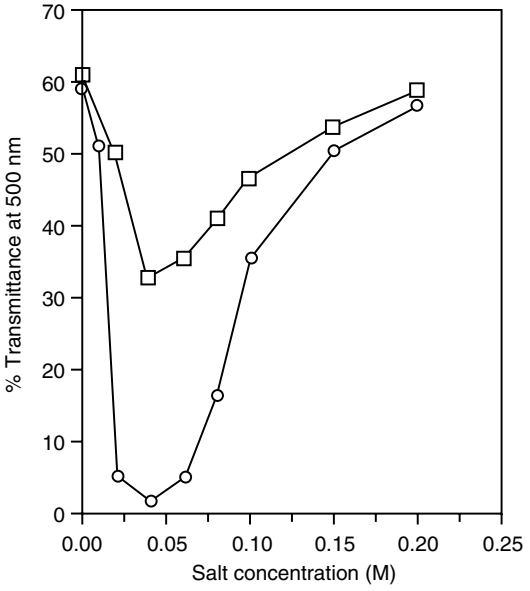


FIGURE 5.34 Salt concentration vs. turbidity of whey protein isolate (5%) in $CaCl_2$ (○) and $MgCl_2$ (□) solutions after incubating for 24 h at ambient temperature. (From Zhu, H. and S. Damodaran. 1994. *J. Agric. Food Chem.* **42**:856–862.)

β-elimination of cystine residues. The resulting increase in the electronegativity and breakage of disulfide bonds causes gross structural changes in proteins exposed to alkali. Generally, alkali-treated proteins are more soluble, and possess improved emulsification and foaming properties.

Hexane is often used to extract oil from oilseeds, such as soybean and cottonseed. This treatment invariably causes denaturation of proteins in the meal, and thus impairs their solubility and other functional properties.

The effects of heat treatments on chemical changes in, and functional properties of, proteins are described in Section 5.6. Scission of peptide bonds involving aspartyl residues during severe heating of protein solutions liberates small molecular-weight peptides. Severe heating under alkaline and acid pH conditions also causes partial hydrolysis of proteins. The amount of small molecular-weight peptides in protein isolates can affect their functional properties.

5.9 CHEMICAL AND ENZYMATIC MODIFICATION OF PROTEINS

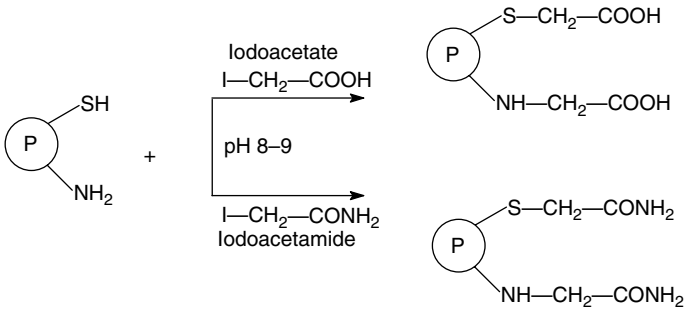
5.9.1 Chemical Modifications

The primary structure of proteins contains several reactive side chains. The physicochemical properties of proteins can be altered and their functional properties can be improved by chemically modifying the side chains. However, it should be cautioned that although chemical derivatization of amino acid side chains can improve functional properties of proteins, it can also impair nutritional value, create some amino acid derivatives that are toxic, and pose regulatory problems although similar reactions may occur *in vivo* or *in situ*.

Since proteins contain several reactive side chains, numerous chemical modifications can be achieved. Some of these reactions are listed in Table 5.5. However, only a few of these reactions may be suitable for modification of food proteins. The ε-amino groups of lysyl residues and the SH group of cysteine are the most reactive nucleophilic groups in proteins. The majority of chemical modification procedures involve these groups.

5.9.1.1 Alkylation

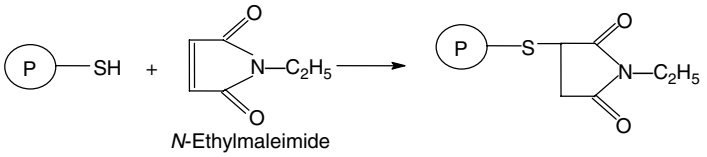
The SH and amino groups can be alkylated by reacting with iodoacetate or iodoacetamide. Reaction with iodoacetate results in elimination of the positive charge of the lysyl residue and introduction of negative charges at both lysyl and cysteine residues.



(5.113)

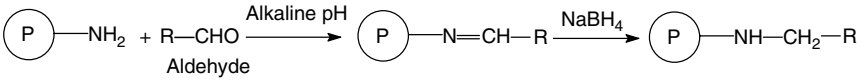
The increase in the electronegativity of the protein may alter the pH-solubility profile of proteins, and may also cause unfolding. On the other hand, reaction with iodoacetamide results only in elimination of positive charges. This will also cause a local increase in electronegativity, but the number of negatively charged groups in proteins will remain unchanged. Reaction with iodoacetamide effectively blocks sulfhydryl groups so disulfide-induced protein polymerization cannot occur.

Sulfhydryl groups also can be blocked by reaction with *N*-ethylmaleimide (NEM).



(5.114)

Amino groups can also be reductively alkylated with aldehydes and ketones in the presence of reductants, such as sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaCNBH_3$). In this case, the Schiff base formed by reaction of the carbonyl group with the amino group is subsequently reduced by the reductant. Aliphatic aldehydes and ketones or reducing sugars can be used in this reaction. Reduction of the Schiff base prevents progression of the Maillard reaction, resulting in a glycoprotein as the end product (reductive glycosylation).

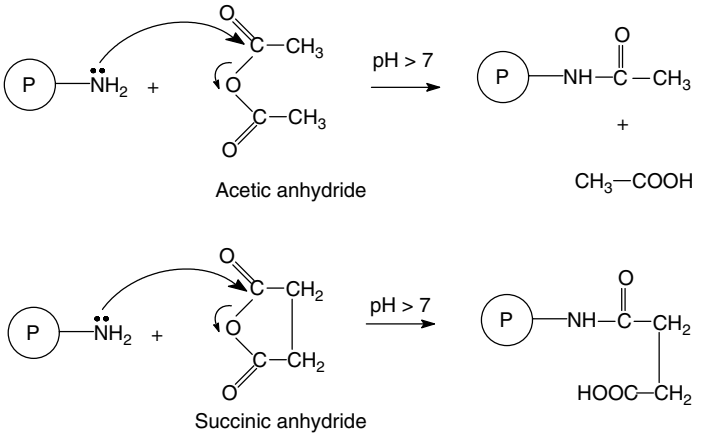


(5.115)

The physicochemical properties of the modified protein will be affected by the reactant used. Hydrophobicity of the protein can be increased if an aliphatic aldehyde or ketone is selected for the reaction and changing the chain length of the aliphatic group can vary the degree of hydrophobicity. On the other hand, if a reducing sugar is selected as the reactant, then the protein will become more hydrophilic. Since glycoproteins exhibit superior foaming and emulsifying properties (as in the case of ovalbumin), reductive glycosylation of proteins should improve solubility and interfacial properties of proteins.

5.9.1.2 Acylation

The amino groups can be acylated by reacting with several acid anhydrides. The most common acylating agents are acetic anhydride and succinic anhydride. Reaction of protein with acetic anhydride results in elimination of the positive charges of lysyl residues, and a corresponding increase in electronegativity. Acylation with succinic or other dicarboxylic anhydrides results in replacement of positive change with a negative change at lysyl residues. This increases the electronegativity of proteins and unfolding of the protein if extensive reaction is allowed to occur.

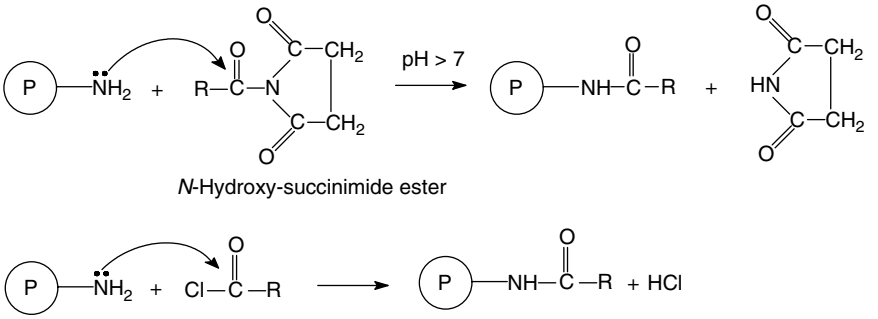


(5.116)

Acylated proteins are generally more soluble than native proteins. In fact, the solubility of caseins and other less soluble proteins can be increased by acylation with succinic anhydride. However, succinylation, depending on the extent of modification, usually impairs other functional properties. For example, succinylated proteins exhibit poor heat-gelling properties, because of the strong electrostatic repulsive forces. The high affinity of succinylated proteins for water also lessens their adsorptivity at oil–water and air–water interfaces, thus impairing their foaming and emulsifying properties. Also, because several carboxyl groups are introduced, succinylated proteins are more sensitive to calcium induced precipitation than is the parent protein.

Acetylation and succinylation reactions are irreversible. The succinyl–lysine isopeptide bond is resistant to cleavage catalyzed by pancreatic digestive enzymes. Furthermore, the intestinal mucosa cells poorly absorb succinyl–lysine. Thus, succinylation and acetylation greatly reduce the nutritional value of proteins.

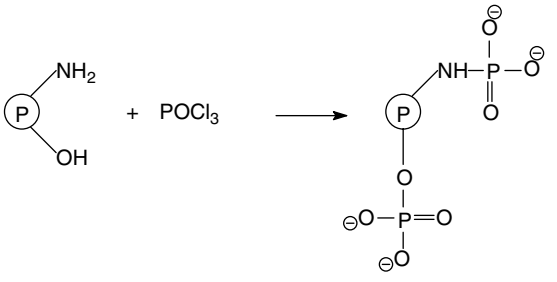
Attaching long chain fatty acids to the ε-amino group of lysyl residues can increase the amphiphilicity of proteins. This can be accomplished by reacting a fatty acylchloride or *N*-hydroxy-succinimide ester of a fatty acid with a protein. This type of modification can enhance lipophilicity and fat binding capacity of proteins; and can also facilitate formation of novel micellar structures and other types of protein aggregates.



(5.117)

5.9.1.3 Phosphorylation

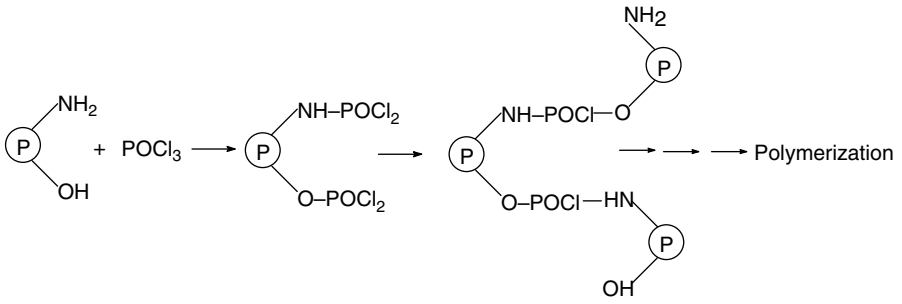
Several natural food proteins, such as caseins, are phosphoproteins. Phosphorylated proteins are highly sensitive to calcium-ion-induced coagulation, which may be desirable in simulated cheese-type products. Proteins can be phosphorylated by reacting them with phosphorus oxychloride, $POCl_3$. Phosphorylation occurs mainly at the hydroxyl group of serine and threonine residues and at the amino group of lysyl residues. Phosphorylation greatly increases protein electronegativity.



(5.118)

Phosphorylation of amino groups results in addition of two negative charges for each positive charge eliminated by the modification. Under certain reaction conditions, especially at high protein concentration, phosphorylation with $POCl_3$ can lead to polymerization of proteins, as shown below. Such

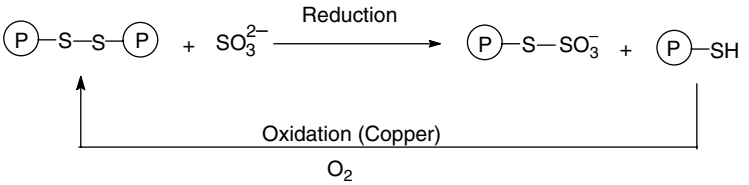
polymerization reactions tend to minimize the increase in electronegativity and calcium sensitivity of the modified protein. The N—P bond is acid labile. Thus, under the conditions prevailing in the stomach, the *N*-phosphorylated proteins would be expected to undergo dephosphorylation and regeneration of lysyl residues. Thus, the digestibility of lysine is probably not significantly impaired by chemical phosphorylation.



(5.119)

5.9.1.4 Sulfitolysis

Sulfitolysis refers to conversion of disulfide bonds in proteins to *S*-sulfonate derivative using a reduction–oxidation system involving sulfite and copper (Cu^{II}) or other oxidants. The mechanism is shown below:



(5.120)

Addition of sulfite to protein initially cleaves the disulfide bond, resulting in the formation of one $S—SO_3^-$ and one free thiol group. This is a reversible reaction, and the equilibrium constant is small. In the presence of an oxidizing agent, such as copper(II), the newly liberated SH groups are oxidized back to either intra- or intermolecular disulfide bonds, and these, in turn, are again cleaved by sulfite ions present in the reaction mixture. The reduction–oxidation cycle repeats itself until all of the disulfide bonds and sulfhydryl groups are converted to *S*-sulfonate derivative [49].

Both cleavage of disulfide bonds and incorporation of SO_3^- groups cause conformational changes in proteins, which affect their functional properties. For example, sulfitolysis of proteins in cheese whey dramatically changes their pH-solubility profiles (Figure 5.35) [48].

5.9.1.5 Esterification

Carboxyl groups of Asp and Glu residues in proteins are not highly reactive. However, under acidic conditions, these residues can be esterified with alcohols. These esters are stable at acid pH, but are readily hydrolysed at alkaline pH.

5.9.2 Enzymatic Modification

Several enzymatic modifications of proteins/enzymes are known to occur in biological systems. These modifications can be grouped into six general categories, namely, glycosylation, hydroxylation, phosphorylation, methylation, acylation, and cross-linking. Such enzymatic modifications of proteins *in vitro* can be used to improve their functional properties. Although numerous enzymatic

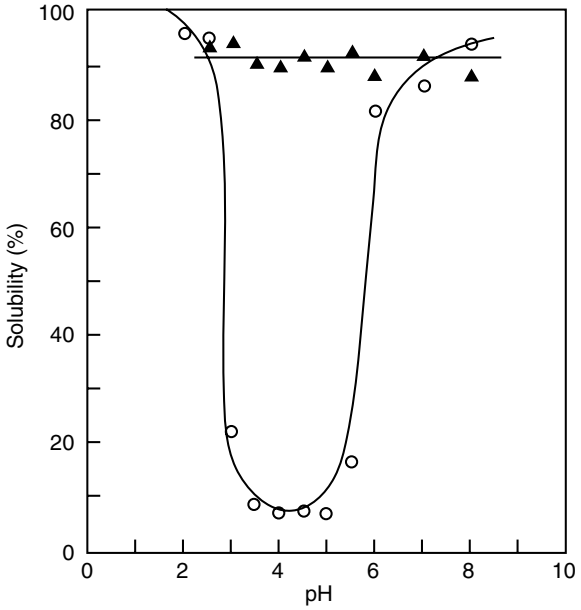


FIGURE 5.35 The pH vs. protein solubility profile of (▲) raw sweet whey and (○) sulfonated sweet whey (From Gonzalez, J. M. and S. Damodaran. 1990. *J. Food Sci*. **55**:1559–1563.)

modifications of proteins are possible, only a few of them are practical for modifying proteins intended for food use.

5.9.2.1 Enzymatic Hydrolysis

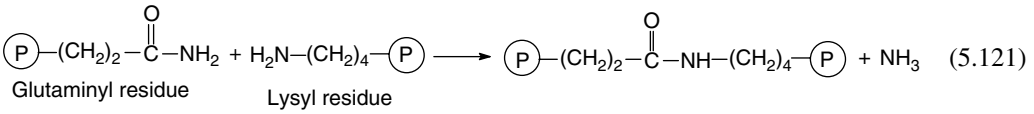
Hydrolysis of food proteins using proteases, such as pepsin, trypsin, chymotrypsin, papain, and thermolysin, alters their functional properties. Extensive hydrolysis by nonspecific proteases, such as papain, causes solubilization of even poorly soluble proteins. Such hydrolysates usually contain low molecular-weight peptides of the order of two to four amino acid residues. Extensive hydrolysis damages several functional properties, such as gelation, foaming, and emulsifying properties (see Section 5.6 for more details).

5.9.2.2 Plastein Reaction

The plastein reaction refers to a set of reactions involving initial proteolysis, followed by resynthesis of peptide bonds by a protease (usually papain or chymotrypsin). The protein substrate, at low concentration, is first partially hydrolysed by papain. When the hydrolysate containing the enzyme is concentrated to 30–35% solids and incubated, the enzyme randomly recombines the peptides, generating new peptide bonds. The plastein reaction also can be performed in a one-step process, in which a 30–35% protein solution (or a paste) is incubated with papain in the presence of L-cysteine [131]. In both cases, however, the molecular weight of the polypeptides formed is typically smaller than the original protein. Thus, the enzyme, especially papain and chymotrypsin, acts both as a protease and an esterase under certain conditions. Since the structure and amino acid sequence of plastein products are different from those of the original protein, they often display altered functional properties. When L-methionine is included in the reaction mixture, it is covalently incorporated into the newly formed polypeptides. Thus, the plastein reaction can be exploited to improve the nutritional quality of methionine or lysine deficient food proteins.

5.9.2.3 Protein Cross-Linking

Transglutaminase catalyses an acyl-transfer reaction that involves reaction between the ε-amino group of lysyl residues (acyl acceptor) and the amide group of glutamine residues (acyl donor), resulting in the formation of an isopeptide cross-link.



(5.121)

This reaction can be used to cross-link different proteins, and to produce new forms of food proteins that might have improved functional properties. At high protein concentration, transglutaminase-catalyzed cross-linking leads to formation of protein gels and protein films at room temperature [85,90,91]. This reaction also can be used to improve nutritional quality of proteins by cross-linking lysine and/or methionine at the glutamine residues (Table 5.23) [58].

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6 Enzymes

Kirk L. Parkin

CONTENTS

6.1 INTRODUCTION

In the 1600–1800s, the action of enzymes in living or respiring tissues were referred to as *ferments*. Examples representing early food enzymology include alcoholic fermentations of yeast, digestive processes in animals, and malting of grains to evoke "diastatic" activity, causing a conversion of starch into sugar. The term "enzyme" was coined by W. Kühne in 1878 from the Greek term *enzyme*, which translates to "in yeast." Thus, early biochemistry was inseparable from what we would now view as food biochemistry.

Food enzymes can be generally classified in two ways: those that are added to foods (exogenous sources) to cause a desirable change and those that exist in foods (endogenous sources) and which may or may not be responsible for reactions that affect food quality. Exogenous enzymes can be obtained from a variety of sources, and choices among exogenous enzymes are based on cost and functionality. Appropriate functionality relates to catalytic activity, selectivity, and stability under the conditions that prevail during the specific application. Endogenous enzymes pose greater challenges to control, since they are present in the food matrix at a range of levels, and there are constraints as to how the foodstuff can be handled to modulate enzyme action. In some foods, endogenous enzymes may be responsible for reactions that either improve food quality or detract from it. The goal of this chapter is to provide the chemical basis for understanding how enzymes function, and how this

understanding can be used to control the action of enzymes for the purposes of transforming foods, producing food ingredients, and maintaining, enhancing, and monitoring food quality.

6.2 GENERAL NATURE OF ENZYMES

6.2.1 Enzymes as Biocatalysts

Enzymes possess three important traits: they are proteins, they are catalysts, and they exhibit selectivity towards substrates. Enzymes are the most common and ubiquitous form of biological catalyst. They are responsible for life processes and mediate synthetic, turnover, signaling, and metabolic functions.

6.2.2 Protein and Nonprotein Nature of Enzymes [28,41,84]

All enzymes are proteins, and proteins are polymers of the naturally occurring L-amino acids. Enzymes range in molecular mass from ~8 kDa (about 70 amino acids, for example, some thioredoxins and glutaredoxins) to 4600 kDa (pyruvate decarboxylase complex). The largest enzymes are comprised of multiple polypeptide chains or subunits and possess quaternary structure. These subunits most often associate through common noncovalent forces (see Chapter 5), and these associations may involve identical (homologous) or dissimilar (heterologous) polypeptide chains. Oligomeric enzymes may possess multiple active sites, and some large enzymes can be comprised of several catalytic activities on a single polypeptide chain. In the latter case, such as the fatty acid synthetase complex of higher organisms, different activities are associated with different protein domains that exist on the polypeptide, and these large polypeptides can associate further as dimers or oligomers. Monomeric enzymes with a single active site can also have different domains within the polypeptide chain, each with a different function related to catalysis or biological properties.

Some enzymes require nonprotein components called "cofactors," "coenzymes," or "prosthetic groups" to carry out their catalytic function [104]. Most common cofactors include metal ions (metalloenzymes), flavins (flavoenzymes), biotin, lipoate, many of the B vitamins, and nicotinamide derivatives (which are really cosubstrates that are bound tightly and undergo reversible redox reactions). Enzymes that are replete with an essential cofactor are called "holoenzymes," while those void of an essential cofactor are called "apoenzymes" and lack catalytic function. Other nonprotein components of enzymes include bound lipid (lipoprotein), carbohydrate (at ASN*, glycoprotein), or phosphate (at SER, phosphoprotein), and while these constituents typically do not have a role in catalysis, they do impact physicochemical properties and confer cellular recognition sites for the enzyme. Enzymes synthesized as latent precursors are referred to as "zymogens" and require proteolytic processing to potentiate their activity (such as digestive enzymes and calf chymosin).

Enzymes existing as monomeric proteins (single polypeptide chain) commonly have molecular masses in the range of 13–50 kDa. The majority of cellular enzymes range in mass between 30 and 50 kDa, and oligomeric enzymes typically range from 80 to 100 kDa being comprised of subunits of 20–60 kDa; only ~1–3% cellular protein is >240 kDa [120]. Oligomeric enzymes are often involved in metabolic processes in the host organism, and the presence of subunits allows for multiple dimensions of regulation by cellular metabolites, allosteric behavior (subunit cooperativity), and interaction with other cellular components or structures.

Extracellular and secreted enzymes tend to be smaller and monomeric polypeptides, often with hydrolytic activities and generally greater stabilities relative to intracellular enzymes. These hydrolytic extracellular enzymes help with mobilizing or assimilating nutrients and growth factors

* Identification of amino acid residues in enzymes will be made using the commonly recognized three-letter codes (upper case). Position in the protein primary sequence, where appropriate, is indicated by subscripts.

from the environment where the (micro)organism would otherwise have little control over factors such as temperature, pH, and composition. Many of the exogenous enzymes used in foods are derived from microorganisms where they can be produced quickly on a large scale by isolation from the fermentation broth. However, enzymes can also be extracted from plant or animal sources and such extracts may be favored in some food applications. Microbial sources of enzymes remain an area of great interest because strain selection and molecular techniques can be used to rapidly select for or modify specific enzyme traits required for certain food processes.

An enzyme can exist as multiple forms that differ slightly in the primary sequence, but possess nearly identical catalytic function. These slight differences in sequence may manifest as subtle or even profound differences in substrate/product selectivity, and characteristic pH and temperature optima. Such entities are referred to as enzyme "isoforms" (less contemporary terms include isozymes and isoenzymes).

On the basis of current wealth of knowledge of protein structure and sequence, enzymes are taxonomically grouped as "families," with members sharing common catalytic function and structural features (with structural elements taking on interesting names such as barrels, propellers, Greek keys, and jelly rolls, terms one may be more likely to hear at a fraternity party than in a discussion of proteins and enzymes). This grouping relates to evolutionary origin and fate. Knowledge of peptide sequence is also instrumental to relating enzymes on the basis of similarity in primary structure (homology), and the presence of small peptide sequences that are "conserved" as "motifs" helps to identify or confirm the existence of the putative active site in mechanistically related enzymes. Understanding how protein structure relates to catalytic function provides the foundation of efforts to improve enzyme use in foods.

6.2.3 Catalytic Power of Enzymes [28,41,52,138]

Catalysts are agents that accelerate the rate of reactions without themselves undergoing any net chemical modification. They also function similarly by reducing the energy barrier required for the transformation of a reactant into a product. This is best illustrated with the use of a hypothetical "reaction coordinate," depicting the free energy change associated with a reaction (Figure 6.1). The progress of a reaction is depicted as stages of transition of a reactant or "substrate" (S) to a product (P). In catalyzed reactions, the substrate (S) is elevated to a transition state ($S^{\ddagger}$) at a reduced expense of free energy ($\Delta G^{\ddagger}_{cat}$) relative to the uncatalyzed reaction ($\Delta G^{\ddagger}_{uncat}$). Figure 6.1 is a simplification since there may be multiple intermediate states in a reaction coordinate. However, there is usually a single, critical or rate-limiting step, either possessing the greatest magnitude or change of $+G$ [145] that generally governs the overall rate for any chemical process. Reactions with a net decrease in free energy ($-\Delta G_{net}$) are favorable, but this does not indicate how *fast* the reaction will go. On the other

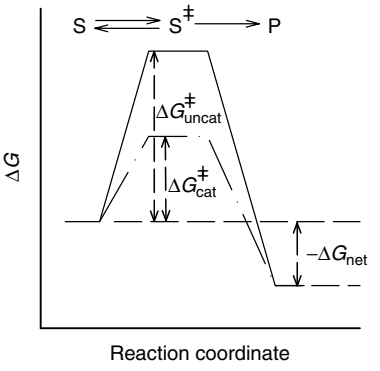


FIGURE 6.1 Comparative reaction coordinates of catalyzed and uncatalyzed reactions.

TABLE 6.1
Examples of Catalytic Power of Enzymes

Reaction	Catalyst	Free Energy of Activation (kcal mol^{-1})	Relative Reaction Rate[a]
$H_2O_2 \longrightarrow \frac{1}{2}O_2 + H_2O$	None (aqueous)	18.0	1.0
	Iodide	13.5	2.1×10^3
	Platinum	11.7	4.2×10^4
	Catalase (1.11.1.6)	5.5	1.5×10^9
p-Nitrophenyl acetate hydrolysis	None (aqueous)	21.9	1.0
	H^+	18.0	7.2×10^2
	OH^-	16.2	1.5×10^4
	Imidazole	15.9	2.5×10^4
	Serum albumin[b]	15.3	6.9×10^4
	Lipoprotein lipase	11.4	5.0×10^7
Sucrose hydrolysis	H^+	25.6	1.0
	Invertase (3.2.1.26)	11.0	5.1×10^{10}
Urea + $H_2O \longrightarrow CO_2 + 2NH_3$	H^+	24.5	1.0
	Urease (3.5.1.5)	8.7	4.2×10^{11}
Casein hydrolysis	H^+	20.6	1.0
	Trypsin (3.4.4.4)	12.0	12.0×10^6
Ethyl butyrate hydrolysis	H^+	13.2	1.0
	Lipase (3.1.1.3)	4.2	4.0×10^6

[a] Relative rates are calculated from $e^{-E_a/RT}$ (Equation 6.1) at 25°C.
[b] Not considered an enzyme reaction.

Source: O'Connor, C.J. and Longbottom, J.R. (1986). *J. Coll. Int. Sci.* 112:504–512; Sakurai, Y., et al. (2004). *Pharmaceut. Res.* 21:285–292; and Whitaker, J.R., et al. (Eds.) (2003). *Handbook of Food Enzymology*, Marcel Dekker, New York.

hand, the reaction rate is dictated thermodynamically by $\Delta G^{\ddagger}$. Examples of the catalytic power of selected enzymes are summarized in Table 6.1 [81,105,141].

Terminology relating to the rate at which an enzyme catalyzes a reaction has been standardized for the purpose of avoiding ambiguity and arbitrary descriptors [3]. One international unit (U) of enzyme activity is defined as that which causes the conversion of 1 μmol substrate per minute under standardized (usually optimized) conditions. The SI unit for enzyme activity is the *katal*, which is defined as the amount of enzyme causing 1 mol substrate conversion per second under defined conditions. Molecular activity of enzymes is defined as a "turnover number" (k_{cat}), or the number of substrate molecules that can be converted by one enzyme molecule (active site) per minute under defined conditions. The upper limit of k_{cat} observed for enzymes is $\sim 10^7$.

6.2.3.1 Collision Theory for Reaction Catalysis

There are two approaches to quantitatively account for rates of chemical reactions (kinetics) and catalysis. The simplest one is the *collision theory*, which is expressed as

$$k = PZe^{-E_a/RT} \tag{6.1}$$

where k is the reaction rate constant, P is the probability of reaction (includes molecular orientation as a factor), Z is the collision frequency, and the exponential term relates to the proportion of colliding reactants having sufficient energy of activation (E_a) to allow reaction. R is the gas constant and

T is the temperature. The most important factor dictating reaction rates as a function of temperature in this equation is the exponential term, as a 10°C increase yields only a ~4% increase in "Z," but a 100% increase (doubling) of the $e^{-E_a/RT}$ term if E_a is 12 kcal mol^{-1} (E_a of enzyme reactions often range 6–15 kcal mol^{-1} [113]). The relationship depicted in Equation 6.1 was developed empirically by S. Arrhenius in the late 1800s, and finds greatest utility in integrated form where enzyme response to temperature can be quantitatively assessed (Section 6.4.1).

6.2.3.2 Transition-State Theory of Enzyme Catalysis

The other, and mechanistically more meaningful, approach to accounting for rates of enzyme reactions is based on the transition-state theory of absolute reaction rates. This theory is largely attributed to H. Eyring (1930s) and is based on the premise that for a reaction of a substrate (S) to product (P) to occur, ground state S must reach an "activated" or transition state ($S^{\ddagger}$), upon which it becomes *committed* to forming P (Figure 6.1). The distribution of S and $S^{\ddagger}$ is characterized by a pseudo-equilibrium constant ($K^{\ddagger}$) as

$$K^{\ddagger} = S^{\ddagger}/S \tag{6.2}$$

and the rate of reaction or decomposition of $S^{\ddagger}$ to P is characterized as

$$dP/dt = k_d[S^{\ddagger}] \tag{6.3}$$

where k_d is the first-order rate constant for the decay of $S^{\ddagger}$ to P. The important thermodynamic parameter is the activation free energy change ($\Delta G^{\ddagger}$) between S and $S^{\ddagger}$ as

$$\Delta G^{\ddagger} = -RT \ln K^{\ddagger} \tag{6.4}$$

Combining equivalencies from Equations 6.2 and 6.4 yields

$$[S^{\ddagger}] = [S]e^{-\Delta G^{\ddagger}/RT} \tag{6.5}$$

The rate constant k_d (Equation 6.3) is equivalent to the vibrational frequency (ν) of the bond undergoing transformation. This is based on the assumption that a molecule in the transition state is so weakened that decay will occur with the next bond vibration [52]. Decay of $S^{\ddagger}$ occurs when the bond vibrational energy is equal to the potential energy, and the relationship becomes

$$k_d = \nu = k_B T/h \tag{6.6}$$

where k_B is the Boltzmann constant and h is Planck's constant. Thus, the theory holds that all transition states decompose at the same rate, and reaction rate is only influenced by [S], temperature, and the characteristic $\Delta G^{\ddagger}$ (which defines $K^{\ddagger}$, Equation 6.4) for an enzyme reaction with a specific S. After substituting for k_d from Equation 6.6 and for $S^{\ddagger}$ from Equation 6.5, the rate Equation 6.3 now becomes

$$\text{rate} = dP/dt = k_S[S] = k_B T/h \times [S]^{-\Delta G/RT} \tag{6.7}$$

Thus, over a range of fixed [S] the reaction rate and rate constant k_S ($k_S[S] = k_B T/he^{-\Delta G^{\ddagger}/RT}$) can be experimentally determined, and then $\Delta G^{\ddagger}$ can be calculated. Once $\Delta G^{\ddagger}$ is determined, the equation can be rearranged to permit the calculation of the thermodynamic entities, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

If one knows the reduction in activation free energy that is afforded by a catalyst, one can quantify or predict the extent to which the reaction is accelerated, based on the collision theory (Equation 6.1)

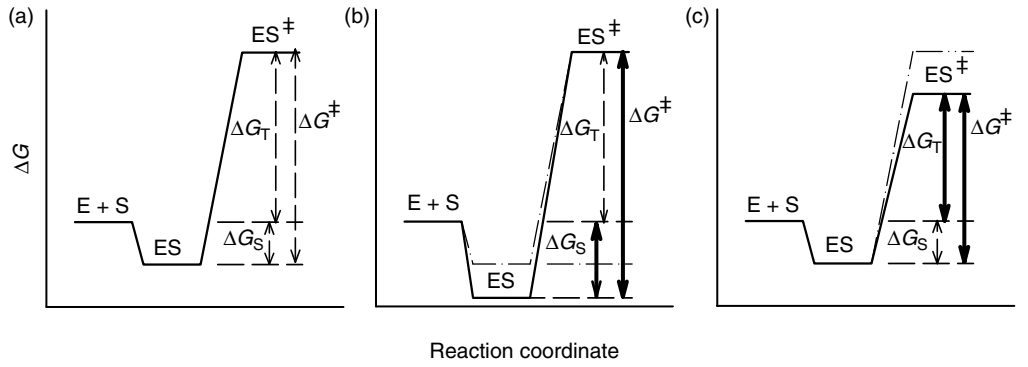


FIGURE 6.2 Reaction coordinate of enzyme reaction and evolutionary advantage. (a) Typical enzyme reaction. (b) Consequence of enzyme evolving to become more complementary to ground state of substrate (S). (c) Consequence of enzyme evolving to be complementary to transition state form of S. Bold arrows denote where changes in ΔG are evident relative to panel (a). (Adapted from Fersht, A. (1985). *Enzyme Structure and Mechanism*, 2nd edn., W.H. Freeman & Company, New York.)

or transition-state theory (Equation 6.7) since the result will be the same and is conferred by the exponential free energy term. For example, if an enzyme reduces the energy of activation ($G^{\ddagger}$ or E_a) of a chemical reaction by 5.4 kcal mol^{-1}, which is quite modest, then the relative rate of the enzyme reaction is accelerated by a factor of 250,000 over the noncatalyzed reaction.

The power of the transition-state theory lies in its simplicity in explaining how enzymes function mechanistically, how they evolve to become more efficient catalysts, and how enzymes are distinguished from antibodies (both selectivity recognize ligands). In the context of enzyme catalysis, free substrate (S) must first bind to free enzyme (E) to yield an association complex that is distributed between ground state (ES) and activated state (ES‡). The role of enzyme is to reduce the $\Delta G^{\ddagger}$ and hence, enhance $K^{\ddagger}$, or the steady-state proportion of S as the activated species S‡, compared to an uncatalyzed reaction. This is indicated for catalysis in general in Figure 6.1, but some key features of enzyme catalysis by transition-state stabilization are better illustrated in a modified reaction coordinate (Figure 6.2a [41]). The association of E and S to form ES has a characteristic free energy of binding (ΔG_S) (often negative for single substrate reactions). Regardless of the magnitude of ΔG_S, this association provides for favorable interactions between E and S, referred to simply as "binding energy," which may be used to facilitate catalysis (Section 6.2.4.2). The next step in catalysis is elevation of S to transition state as ES‡ (all of which forms P and free E). This step is thermodynamically represented as $\Delta G^{\ddagger}$. The minimum net activation free energy change for reaction to occur (for free S $\rightarrow$ P) is ΔG_T. ΔG_T is the sum of the free energies of the individual binding (ΔG_S) and catalytic ($\Delta G^{\ddagger}$) steps. Using this diagram, it becomes easy to see where the catalytic advantage lies for enzymes as they evolve to recognize substrates. If the enzyme binding site for substrate evolves *only* in a manner to better recognize (become more complementary to) the ground state of S, affinity between E and S will improve and binding becomes more favorable (more negative ΔG_S; Figure 6.2b). The consequence is there is no change in ΔG_T, but there is an increase in $\Delta G^{\ddagger}$, and a larger energy barrier must be overcome for the step ES $\rightarrow$ ES‡. Alternatively, if the *only* change in enzyme–substrate recognition is that the binding site becomes more complementary to the structure represented by S‡, then the free energy for both the net reaction (ΔG_T) and the bond-making/breaking step ($\Delta G^{\ddagger}$) is reduced (Figure 6.2c). It should be clear that the advantage lies in the enzyme recognizing or stabilizing the transition state form of S.*

* The enzyme does not recognize S‡ existing in solution, but rather, S becomes converted to, or stabilized as, S‡ on binding, through utilization of some of the binding energy and the mechanistic forces involved in enzyme catalysis.

6.2.4 Mechanisms of Enzyme Catalysis [28,41,138]

On the molecular level, enzymes possess active sites that bind S and stabilize $S^{\ddagger}$. Amino acid residues that form the active site and any required cofactors collectively interact with substrate via covalent and/or noncovalent interactions. Enzymes may use a number of mechanisms to catalyze bond-making/breaking and atomic rearrangement processes, and the ability to do this is founded on the specific amino acids and their spatial arrangement within the active site. Aside from the amino acids *essential* for catalysis, other amino acids may *assist* in catalysis through S recognition and $S^{\ddagger}$ stabilization.

6.2.4.1 General Nature of Enzyme Active Sites

Certain amino acids of enzymes are responsible for catalytic activity. Considering the size of proteins, it may seem surprising that only a limited number of amino acids, typically ranging from 3 to 20, are responsible for catalytic function [120], with the number being somewhat proportional to the size of the enzyme. On the other hand, the group of enzymes known as the serine proteases range in size from 185 to 800 amino acid residues, corresponding to 20–90 kDa (most are 25–35 kDa), but contain the same catalytic unit (triad) of HIS–ASP–SER. These comparisons illustrate that enzymes contain many more amino acid residues than are required for catalytic activity, and this prompted the question, "why are enzymes so big" [120]. The catalytic amino acid residues of enzymes are rarely proximal to each other in the primary sequence and are distributed throughout the polypeptide chain. For example, the catalytic triad is HIS_{64}–ASP_{32}–SER_{221} for the *Bacillus subtilis* protease subtilisin, and HIS_{257}–ASP_{203}–SER_{144} for the *Rhizomucor miehei* lipase (serine proteases and lipases are mechanistically related) [20,59]. Thus, one function of the noncatalytic portions of the polypeptide chain is to bring the catalytic residues into the same three-dimensional space by virtue of the protein's secondary and tertiary structure. The precise spatial arrangement of the catalytic residues allows them to function as a catalytic unit, and polypeptide folding also brings together other sets of residues possessing a set of characteristic binding forces to afford substrate recognition. Thus, polypeptide conformation acts as a "scaffold" to correctly position, within a three-dimensional space, the amino acid residues with catalytic and substrate-recognizing functions.

Another role of the polypeptide chain is to provide for close packing of atoms, such that water is largely excluded within the enzyme interior [41]. Limiting water to 25% of the protein volume allows for interior cavities and clefts to form that are relatively nonpolar and devoid of water, and this can enhance dipole forces in facilitating catalysis. Other noncatalytic amino acid residues may participate in overall enzyme functioning by serving as cofactor or affector binding sites, surface recognition sites for interaction with other cellular components or to attract/trap substrate [41,120]. Lastly, amino acids not involved in catalysis or substrate recognition may dictate sensitivity of the protein conformation to environmental factors such as pH, ionic strength, and temperature, such that they modulate enzyme activity and confer overall enzyme stability.

6.2.4.2 Specific Catalytic Mechanisms

Mechanisms for how enzymes function as catalysts can be reduced to about four general categories [28,52]. These are approximation, covalent catalysis, general acid–base catalysis, and molecular strain or distortion (Table 6.2 [28,104,138]). Other forces that contribute to catalysis will be identified where appropriate.

6.2.4.2.1 Role of Binding Energy

Before describing each of the major enzyme mechanisms, it is necessary to expand on the role of binding energy, which was introduced in Section 6.2.3.2, as it contributes to all of the mechanisms described hence. Binding energy is the term used to refer to the favorable interactions derived

TABLE 6.2
Common Mechanisms of Enzyme Catalysis

Mechanism	Forces Involved	Residues and Cofactors Potentially Involved
Approximation	Modeled as intra- vs. intermolecular catalysis	Active site and substrate-recognizing residues
Covalent catalysis	Nucleophilic	SER, THR, TYR, CYS, HIS (base), LYS (base), ASP^-, GLU^-
	Electrophilic	LYS (Schiff-base), pyridoxal, thiamine, metals (cations)
General acid–base catalysis	Proton association/dissociation, charge stabilization	HIS, ASP, GLU, CYS, TYR, LYS
Molecular strain/distortion	Induced fit, induced strain, rack mechanism, conformational flexibility	Active site and substrate-recognizing residues

Source: Copeland, R.A. (2000). *Enzymes. A Practical Introduction to Structure, Function, Mechanism, and Data Analysis*, 2nd edn., John Wiley & Sons, New York; Saier, M.H. (1987). *Enzyme in Metabolic Pathways. A Comparative Study of Mechanism, Structure, Evolution and Control*, Harper & Row, New York; and Walsh, C. (1979). *Enzymatic Reaction Mechanisms*, W. H. Freeman and Company, San Francisco, CA.

on association of substrate and enzyme at the binding site [28,41,138]; binding energy is derived from the complementary features existing between enzyme and substrate. Complementarity may be "preformed" (founded on the old "lock and key" concept of enzyme–substrate recognition advanced by E. Fischer), or "developed" on binding, or be a combination of the two. Net binding energy is also defined as the free energy change (often negative) resulting from the desolvation of substrate in exchange for interaction with enzyme. The entropy loss due to enzyme–substrate association is offset by entropy gained by solvent (usually water). Some of this binding energy may be used for productive purposes in catalysis: that is, some binding energy can be converted to mechanical and/or chemical activation energy. It can be used to mobilize S at the active site, or it can be used to destabilize S, or stabilize $S^{\ddagger}$. The ability of an enzyme to react faster with one substrate over another (defined as "selectivity") may be directly related to how much binding energy can be used to facilitate the catalytic step. Catalytically nonessential amino acid residues at/near the active site often assist catalysis through the use of binding energy.

6.2.4.2.2 Approximation

Approximation is best described as the catalytic units and substrate being near or proximal to each other in a favorable orientation, facilitating reactivity. Another way to envision the catalytic power of approximation is that since the reactants are localized in the same space within the enzyme active site, their "effective molarity" is greatly enhanced relative to solution concentrations. This mechanism offers the "entropic contribution" to catalysis as it helps to overcome the large decrease in entropy otherwise necessary to bring together all participants in a reaction. Thus, the contribution of approximation effects to catalysis is often modeled by effective (enhanced) concentrations in the context of mass action effects on reaction rates.

The lifetime of intermolecular associations between reactants colliding in solution is typically 6 orders of magnitude shorter than that of a complex formed by typical binding of substrate to enzyme [138]. The enzyme binding pocket affords the "docking," or "anchoring" of substrate at the active site in a water-diminished environment. The longer lifetime of interaction would by itself lead to greater probability of reaching the transition state. Thus, approximation can also be modeled as an

*intra*molecular reaction, where all reactants are viewed as existing *within* a single molecule (the enzyme), compared to an *inter*molecular reaction.

The net catalytic effect of approximation is based on rather theoretical calculations, but is viewed as yielding up to a 10^4–10^{15} rate enhancement over a chemical reaction involving one to three substrates (greater enhancement for multiple substrate reactions) [138,141]. Approximation is a mechanistic feature that is not conferred by specific amino acids, but rather by the chemical and physical nature of the active site and the constellation of amino acids that comprise it (Table 6.2).

6.2.4.2.3 Covalent Catalysis

Covalent catalysis involves the formation of an enzyme–substrate or cofactor–substrate covalent intermediate, and this mechanism of catalysis is initiated by nucleophilic or electrophilic attack. (*Nucleophilic and electrophilic behavior of enzyme residues/cofactors may also be involved in non-covalent mechanisms*.) Nucleophilic centers are rich in electrons—or possess unpaired electrons and are sometimes negatively charged—and they seek electron-deficient centers (nuclei) with which to react, such as carbonyl carbons, or phosphoryl or glycosyl functional groups. Electrophilic catalysis involves the withdrawal of electrons from reaction centers by electrophiles, also referred to as electron "sinks." While covalent catalysis involves both nucleophilic and electrophilic groups among the reactants, the classification of the reaction as one or the other is based on which center is initiating the reaction.

Implicit with the formation of a covalent intermediate is the existence of at least two steps along the reaction coordinate, namely the formation and breakdown of the covalent adduct (Enz–Nu–P_2), each with a characteristic $\Delta G^{\ddagger}$ (Figure 6.3). The multiple stages of catalysis also reflect the presence of multiple enzyme forms, posing a kinetically more complicated reaction coordinate than is depicted in Figure 6.1. Covalent catalysis is common to many classes of enzymes, including the serine and thiol proteases, lipases and carboxylesterases, and many glycosyl hydrolases. The net catalytic effect of covalent catalysis is estimated as yielding up to a 10^2–10^3 rate enhancement over a chemical reaction.

Nucleophilic catalysis: Amino acid residues of enzymes that provide nucleophilic centers are listed in Table 6.2. Generally, nucleophilicity is dependent on basicity of the functional group, which relates to the ability to donate an electron pair to a proton [28,41]. Thus, the nucleophilic rate constant is

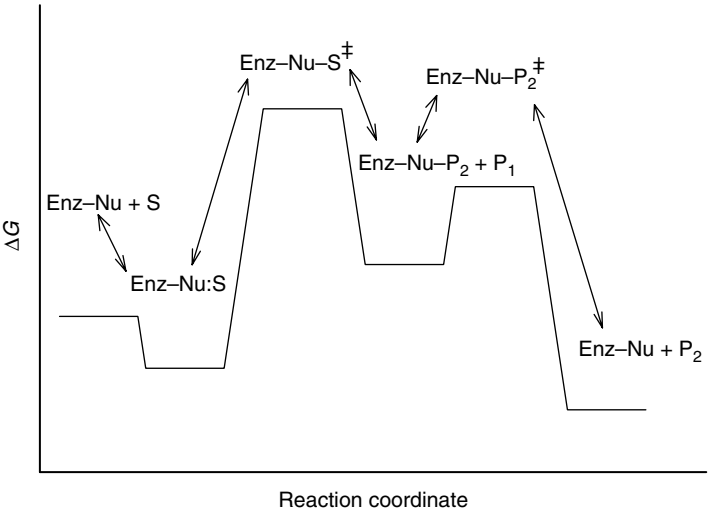


FIGURE 6.3 Reaction coordinate for enzyme reaction by nucleophilic catalysis with covalent intermediate. Enz–Nu = enzyme with nucleophilic catalytic group; S = substrate; P_x = products.

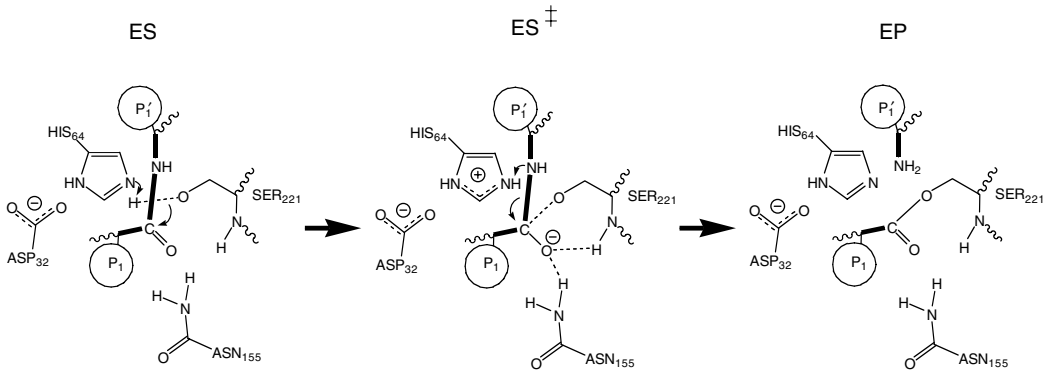


FIGURE 6.4 Reaction mechanism of serine proteases. Substrate peptide backbone in bold. P_1 and P_1' groups denote the side chains of amino acid comprising the respective N- and C-terminal sides of the scissile bond. (Adapted from Carter, P. and Wells, J.A. (1988). *Nature* 332:564–568 and Carter, P. and Wells, J.A. (1990). *Prot. Struct. Func. Genet.* 7:335–342.)

correlated positively with the pK_a for structurally related compounds (greater pK_a's yield greater reaction rates). However, nucleophilic groups in enzymes must often function over a limited range of pH (often at pH near 7) that is conducive to maintaining enzyme conformational stability. Thus, while ARG provides for a strongly basic functional group, its pK_a value of ~12 precludes it from acting as a nucleophilic residue since it would exist almost exclusively as the conjugate acid form in active enzymes, which explains why it is not listed in Table 6.2. One other factor that impacts the rate of nucleophilic catalysis is the nature of the "leaving group" or the products formed during formation of the covalent intermediate (P_1 in Figure 6.3). The weaker the basicity (lesser pK_a value) of the leaving group, the greater the rate of reaction for a given nucleophile.

The catalytic triad HIS–ASP(GLU)–SER characteristic of the serine protease and lipase/carboxylesterase families of enzymes is one of the most studied examples of nucleophilic catalysis. These enzymes catalyze the hydrolysis of amide (peptide) and ester bonds, respectively, via a covalent intermediate. The functioning of the HIS–ASP–SER catalytic unit also illustrates that while one process (nucleophilic catalysis in this case) may be used to classify a reaction mechanism, several mechanistic forces are often combined during the course of enzymic catalysis. For the catalytic triad of subtilisin (*B. subtilis* protease, EC 3.4.21.62), SER_{221} acts as a nucleophile in seeking to donate electrons to the amide carbon of the peptide bond (Figure 6.4 [20,21]). The nucleophilicity of the SER_{221} oxygen atom is enhanced by HIS_{64} acting as a general base to accept a proton; the neighboring ASP_{32} residue stabilizes the developing charge on the HIS_{64}. This results in the formation of the transient tetrahedral acyl–enzyme intermediate. In the last stage, HIS_{64} acts as a general acid to donate a proton to the N-terminal peptide fragment of the cleaved peptide, which constitutes the leaving group, and the covalent acyl–enzyme adduct is formed. Although not shown in this figure, the completion of the catalytic cycle is achieved when water, acting as a terminal nucleophile, enters the active site and displaces the peptide fragment from SER_{221}, by forming another tetrahedral intermediate using the same catalytic machinery as just described. The ASN_{155} residue is less critical to catalysis but functions to stabilize the developing tetrahedral intermediate (an "oxyanion") within a space in the enzyme referred to as the "oxyanion hole."

The behavior of subtilisin mutants (where specific amino acid residues are replaced by others, using molecular techniques) reveals the importance of the amino acids comprising the triad. The native enzyme has a catalytic efficiency (indexed as k_{cat}/K_M, explained in Section 6.2.5.3) of 1.4×10^5 (Table 6.3 [20,21]). If either of the SER_{221}, HIS_{64} or ASP_{32} residues is replaced by ALA, catalytic efficiency is reduced by about 10^4–10^6. When any two or all three of these residues are replaced with ALA, little or no further compromise in catalytic efficiency is observed, showing that the three

TABLE 6.3
Effect of Point Mutations on Catalytic Constants of Subtilisin Protease

Enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
Wild-type	6.3×10^{1}	440	6.3×10^{5}
$SER_{221} \longrightarrow ALA$	5.4×10^{-5}	650	8.4×10^{-2}
$HIS_{64} \longrightarrow ALA$	1.9×10^{-4}	1300	1.5×10^{-1}
$ASP_{32} \longrightarrow ALA$	1.8×10^{-2}	1400	1.3×10^{1}
All three mutations	7.8×10^{-3}	730	1.1×10^{-1}

Sources: Carter, P. and Wells, J.A. (1988). *Nature* 332:564–568 and Carter, P. and Wells, J.A. (1990). *Prot. Struct. Func. Genet.* 7:335–342.

amino acid residues act as a *unit*, rather than making accretive contributions to catalytic power. These same amino acid residues make up the catalytic triad of lipases (and most carboxylesterases). For lipases, the same sequence of events as depicted in Figure 6.4 takes place, except that the substrate is an ester (R—CO—OR′), where the acyl group (R—CO—) goes on to form the same acyl–enzyme intermediate, while the liberated alcohol (R′OH) constitutes the leaving group. The catalytic triad of HIS–ASP(GLU)–SER is a highly conserved catalytic unit for lipases and carboxylesterases, whereas proteases may work by any of four distinct catalytic mechanisms (Section 6.3.3). Three carboxylesterases that use other catalytic units and mechanisms include secretory phospholipase A_2 (pancreatic, bee, and snake venom; HIS/ASP dyad), potato lipid acyl hydrolase (ASP/SER dyad), and pectin methyl esterase (ASP/ASP dyad).

Electrophilic catalysis: Electrophilic catalysis constitutes another type of covalent mechanism, where the characteristic step in the reaction coordinate is electrophilic attack. Amino acid residues in enzymes do not provide adequate electrophilic groups. Instead, electrophiles are drawn from electron-deficient cofactors or a cationic nitrogen derivative formed between substrate and the enzyme catalytic residues to initiate electrophilic catalysis (Table 6.2).

Some of the best characterized enzyme reactions evoking electrophilic catalysis employ pyridoxal phosphate (an essential vitamin nutrient, B_6, Chapter 7) as a cofactor; many such enzymes are involved in amino acid transformation/metabolism [41,128]. A general mechanism of pyridoxal–enzyme reactions involves transfer (transaldimination) of a Schiff base (—C═N—) linked pyridoxal group from an enzyme–LYS residue to a reactive amino acid bound at the enzyme active site (Figure 6.5a [41,128]). The Schiff base intermediate is stabilized by the pyridine ring that acts as an electron sink. A residue on the enzyme then acts as a base (B:) to absorb the proton liberated from the substrate as a common first step in the reaction pathway. The preference of substituent group about the chiral center (—R, —H, $—COO^-$) to be cleaved ("lysed") or transferred is based on which α-C substituent group is perpendicular to the plane of the pyridinium intermediate as it has the lowest E_a for transformation/removal (Figure 6.5b).

Some of the active site features shared by many pyridoxal-enzymes are illustrated with alliin lyase (EC 4.4.1.4, *S*-alk(en)yl-L-cysteine sulfoxide lyase) action on *S*-alk(en)yl-L-cysteine sulfoxide (Figure 6.6 [64]). This enzyme is commonly referred to alliinase, and is responsible for the generation of characteristic flavors of *Allium* vegetables (onion, garlic, leek, chive, etc.) upon initial disruption or cutting of fresh tissues. For the garlic enzyme, LYS_{251} (LYS_{285} in onion and LYS_{280} in chive) coordinates with the pyridoxal cofactor, aided by the "phosphate binding cup" and additional residues that bind with the pyridinium N and hydroxyl groups [64]. Substrate coordinates with other enzyme residues (ARG_{401}, SER_{63} and GLY_{64} amide, and TYR_{92}) to confer enzyme (stereo)selectivity toward the (+)*S*-alkyl-L-cysteine sulfoxides. Alliinase causes β-cleavage of the substrate, yielding the sulfenic acid (R—S—OH, a good leaving group).

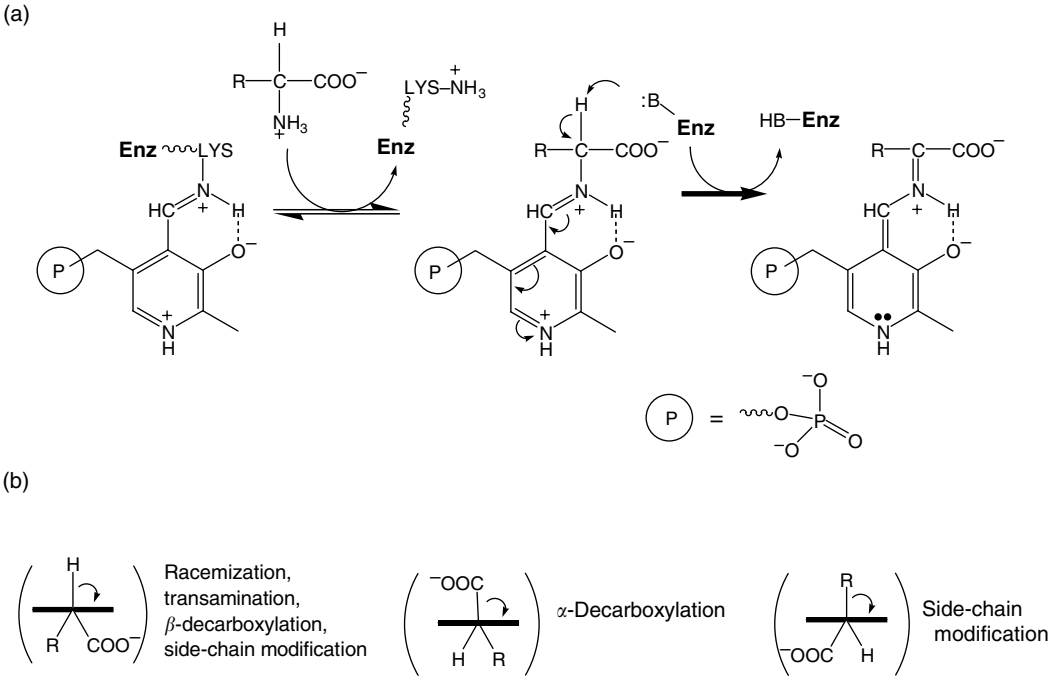


FIGURE 6.5 General reaction mechanism of pyridoxal-containing enzymes. (a) Initial steps of transaldimination and removal of α-H atom. (b) Relationship of α-C configuration to types of reactions catalyzed. (Adapted from Fersht, A. (1985). *Enzyme Structure and Mechanism*, 2nd edn., W. H. Freeman & Company, New York and Tyoshimura, T., Jhee, K.-H. and Soda, K. (1996). *Biosci. Biotech. Biochem.* 60:181–187.)

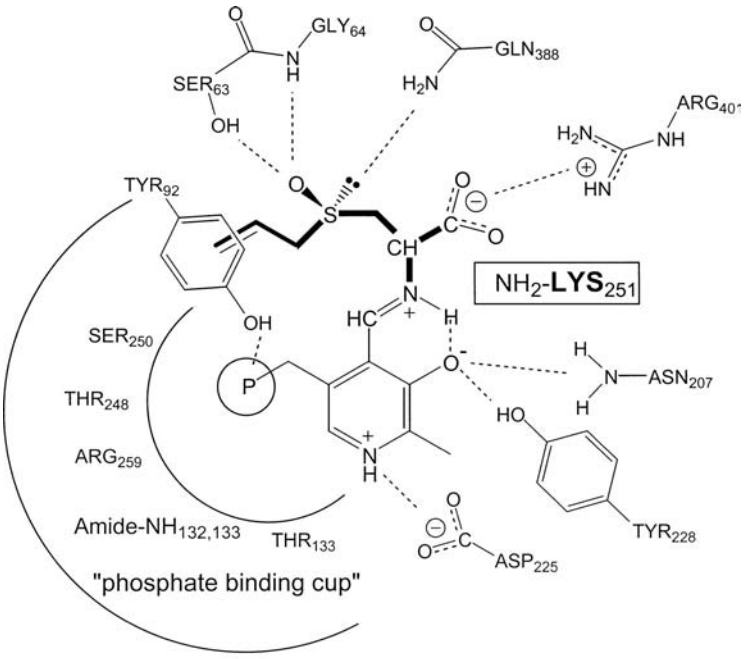
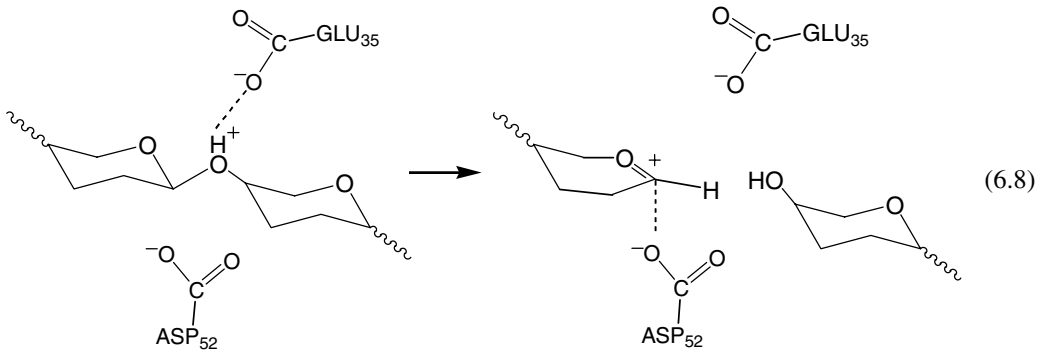


FIGURE 6.6 Active site of garlic alliinase. Backbone of (+)*S*-allyl-L-cysteine sulfoxide substrate in bold. (Adapted from Kuettner, E.B., et al. (2002). *J. Biol. Chem.* 277:46402–46407.)

6.2.4.2.4 General Acid–Base Catalysis

Most enzyme reactions involve proton transfer at some point during catalysis (in reaching transition state) and this is often accomplished by amino acid residues that act as general acids to donate a proton and general bases to accept a proton. General acid–base catalysis can be distinguished from specific acid (H_3O^+) or base (^-OH) catalysis because reactions rates for general acid–base catalysis are dependent on concentration of the catalyst. Enzymes employ *general* acid–base mechanisms since they cannot concentrate H_3O^+ or ^-OH (although some enzymes can generate H^+ or ^-OH at the active site). Amino acid residues on enzymes that can function as general acids–bases typically have pK_a values in the range of the pH optimum for enzyme activity and stability (generally pH 4–10), and such residues appear in Table 6.2. Recall that general acid–base behavior contributes to the nucleophilic mechanism of serine proteases, lipases, and carboxylesterases (Figure 6.4). Indeed, HIS is a residue very often involved in general acid–base catalysis, because the pK_a of the imidazole group within proteins is usually in the range of 6–8, making it ideal for functioning as *either an acid or base* under conditions where many enzymes are active.

An example of general acid–base catalysis is found in lysozyme (EC 3.2.1.17, mucopeptide *N*-acetylmuramyl hydrolase, also called muramidase), an enzyme occurring in saliva, tear duct secretion, and hen's egg white. The mechanism evoked by lysozyme applies to glycosyl hydrolases in general (Section 6.3.2), which include the starch-, sugar-, and pectin-transforming enzymes [116]. Lysozyme may be used as a bacteriocidal agent in foods since it hydrolyzes the peptidoglycan heteropolymers of prokaryotic cell walls (especially of Gram-positive microorganisms, which include many food pathogens). Best illustrated at the near-optimum pH ~5, the mechanism of action relies on the general acid–base nature of active site amino acids GLU_{35} and ASP_{52} [32,116,141].



(6.8)

The proton of GLU_{35} acts as a general acid and coordinates with the oxygen atom of the scissile glycosidic bond; ASP_{52} carboxylate serves to electrostatically stabilize the developing carboxenium ion of the substrate by acting as a base.* The incoming water needed to complete hydrolysis (not shown) is partially ionized by the GLU_{35} carboxylate group, to activate the addition of —OH (from water) to C1 of the original glycoside, with H^+ acquired by GLU_{35} to return the enzyme to the active state. The exclusion of water and an abundance of hydrophobic residues at the active site cleft of the enzyme create a nonpolar environment proximal to the GLU_{35} residue, rendering it less capable of ionizing and conferring an abnormally high pK_a of 6.1. This allows it to function as a general acid catalyst at pH 5. The relative lack of water to shield charges also allows for fixed dipoles to emerge between the catalytic residues and the developing carboxenium ion intermediate. This serves to

* Many glycosyl hydrolases, including lysozyme, are classified as examples of nucleophilic catalysis because a covalent intermediate is formed [116] although not shown in Equation 6.8. The ASP_{52} carboxylate is a good nucleophile (Table 6.2). Glycosyl hydrolase mechanisms are fully explained in Section 6.3.2.

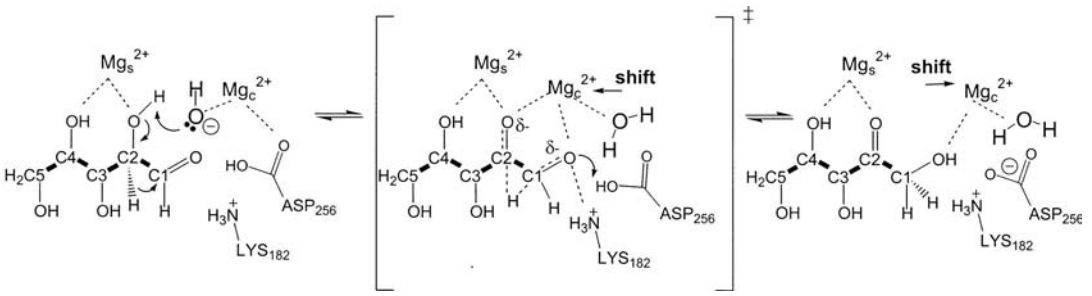


FIGURE 6.7 Reaction mechanism of xylose (glucose) isomerase. (Adapted from Garcia-Viloca, M., et al. (2002). *J. Am. Chem. Soc.* 124:7268–7269 and Garcia-Viloca, M., et al. (2004). *Science* 303:186–195.)

reduce E_a by ≥9 kcal/mol (corresponding to a rate enhancement of $>10^6$) relative to the uncatalyzed reaction in water [32].

An example of proton/hydrogen transfer reactions in enzymes is found in xylose isomerase (EC 5.3.1.5, D-xylose ketol isomerase), also referred to as glucose isomerase. This enzyme catalyzes an equilibrium reaction between aldose and ketose isomers. Almost all xylose isomerases characterized are homotetramers, yielding two active sites each with a cation cofactor (commonly Mg^{2+}; also Mn^{2+}, Co^{2+}) [141]. A conserved active site sequence (*Streptomyces* spp. as reference) includes residues binding the cations ($GLU_{180,216}$, $ASP_{244,254,256,286}$, HIS_{219}) and others lining the active site (HIS_{53}, PHE_{93}, TRP_{135}, LYS_{182}, GLU_{185}) [116]. The active site is bifurcated with highly polar and hydrophobic areas (especially TRP_{135}), the latter serving to exclude water. This enzyme has historically been cited as an example of general acid–base catalysis, but a more contemporary view is that it catalyzes a hydride transfer reaction. The specific steps in the reaction sequence include ring opening, rate-limiting hydrogen transfer step, and ring closure [46,47]. Of the two Mg^{2+} ions, Mg_s is structural and coordinates with O2 and O4 of the sugar substrate, and Mg_c is catalytic (Figure 6.7 [46,47]). After ring opening (not shown), $^-$OH is generated from water by ASP_{256} carboxylate acting as a general base to remove a H^+. A proton from O2 is transferred to $^-$OH bound to Mg_c, after which Mg_c is then drawn to the negatively charged O2 (Mg_c actually *moves*) to stabilize the transition state, and this is assisted by H-bonding between LYS_{182} and O1. This movement of Mg_c is synchronous with the transfer of the hydride ($^-$H:) from C2 to C1. This is an equilibrium reaction, and hydride transfer can be reversed by essentially the same steps with the Mg_c : $^-$OH coordinate serving to shuttle H^+ from O1 to O2 alkoxides to facilitate hydride transfer from C1 to C2.

Reaction rate enhancements of 10^2–10^3 are typically contributed by general acid–base catalysis, where the pushing or pulling of electrons is required along the reaction coordinate. In the example of lysozyme, the much greater overall rate enhancement is based on other factors (electrostatic stabilization and substrate strain) contributing to catalysis.

6.2.4.2.5 Strain and Distortion

This mechanistic explanation is founded on the premise that interacting domains of substrates and enzymes are not as rigid as implied by the "lock-and-key" conceptualization of enzyme catalysis advanced by E. Fischer in 1894. Distortion or strain as a factor in governing catalysis was offered by J.B.S. Haldane and L. Pauling, as it related to the transition-state theory of enzyme catalysis. Thus, while there is structural complementarity between enzyme and substrate to provide for attractive forces, this complementarity is not "perfect." If "preformed" complementarity was "perfect," catalysis would less likely take place because of the large energy barrier required to reach a transition state (recall Figure 6.2b).

Some preformed complementarity between enzyme and substrate binding site provides for substrate recognition, the acquisition of binding energy, and helps orient substrate at the active site. The

productive utilization of binding energy arising from enzyme–substrate association may manifest as inducing stress/strain on the enzyme and/or substrate, allowing for complementarity to be further "developed." The effects on substrate are unlikely to involve bond stretching, twisting, or bending of bond angles because of the large forces estimated to be required for such events [41]. Rather, strain on the substrate more likely occurs as restriction in bond rotational freedom, steric compression, and electrostatic repulsion between enzyme and substrate. Thus, in a true physical sense, substrate may be subjected to "stress" (where distortion does not occur) upon binding to enzyme in a manner that the relief of that stress through utilization of some binding energy helps promote the transition state. An example of this is found in the mechanism of lysozyme, where the transition-state carboxenium ion of the pyranose derivative (Equation 6.8) assumes a half-chair ("sofa") instead of the more stable full-chair conformation.

Enzymes as proteins are considered to possess more flexible structures than small (in)organic substrates. In contrast to preformed complementarity, protein conformational flexibility provides the basis for the "induced-fit" hypothesis for enzyme catalysis originally advanced by D. Koshland. Here, conformational perturbations in the enzyme active site upon substrate binding are viewed as facilitating the stabilization of the $ES^{\ddagger}$ complex. In doing so, the conformational modulation in the enzyme active site *upon binding of substrate* may help align reactive groups of both enzyme and substrate to facilitate catalysis.

One example of an induced fit mechanism of catalysis is the surface activation of lipases, where a protein domain constituting a "lid" covering the active site undergoes a conformation shift to allow the fatty acid ester substrate to gain access to the active site and undergo hydrolysis. A more subtle molecular motion in enzymes involves the movement of Mg_c in the xylose isomerase just described (Figure 6.7), the estimated acceleration of reaction rate from which is about 10^4 [47]. A third example of induced fit is papain, a sulfhydryl protease, in which sterically induced strain upon binding of substrate is relieved upon formation of a tetrahedral intermediate; specificity and mechanism of papain are featured later in Sections 6.2.6 and 6.3.3. It is becoming apparent that many, if not most, enzymes evoke induced fit to some degree during catalytic function. While estimates of the net catalytic effect of strain are difficult to quantify, the extent of rate acceleration is in the range of 10^2–10^4.

6.2.4.2.6 Other Enzyme Mechanisms

Redox enzymes (oxidoreductases) catalyze electron transfer reactions by cycling between redox states of prosthetic groups. Prosthetic groups can be transition metals (iron or copper), or cofactors such as flavins (nicotinamides, such as NAD(P)H, are cosubstrates in redox reactions). Lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12) is widely distributed in plants and animals, and possesses nonheme iron as the prosthetic group. It is reactive with fatty acids having a 1,4-pentadiene group of polyunsaturated fatty acids (there may be multiple such groups in fatty acids), represented by linoleic acid ($18{:}2_{9c,12c}$). Lipoxygenases initiate oxidative degradation of fatty acids into products that collectively can impart either undesirable (rancid) or desirable flavors, and they can also bleach pigments through secondary reactions. Lipoxygenase is often isolated from host tissues in the "inactive" $Fe^{(II)}$ state (Figure 6.8 [14,22]). Activation occurs by reaction with a peroxide (there are low levels of peroxides existing in all biological tissues), yielding the activated $HO–Fe^{(III)}$ complex with the coordinated hydroxide group serving as the base to abstract an H atom (through a process called "tunneling"*) from the methylenic carbon (these C—H bonds have the lowest bond energy in fatty acids and are thus easiest to react). The free radical adduct is resonance stabilized, and O_2 adds to the alkyl radical at permitted sites at the opposite side of the substrate from Fe (see later discussion on specificity, Section 6.2.6). The resulting peroxyl radical abstracts a H atom from the

* Tunneling is a mechanism (modeled as a transmission coefficient) to describe H transfer when less energy is required than expected (a shortcut or tunnel is "dug" under the energy barrier). This may involve H transfer in two inseparable parts, first the nuclei followed by the electron [47].

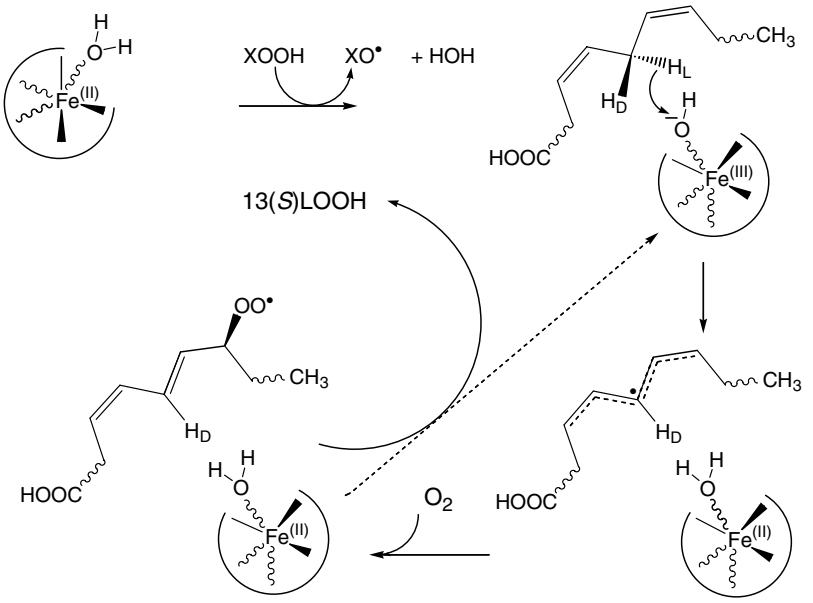


FIGURE 6.8 Reaction mechanism of lipoxygenase. (Adapted from Brash, A.R. (1999). *J. Biol. Chem.* 274:23679–23682; Casey, R. and Hughes, R.K. (2004). *Food Biotechnol.* 18:135–170; and Sinnott, M. (Ed.) (1998). *Comprehensive Biological Catalysis. A Mechanistic Reference*, Vol. III, Academic Press, San Diego, CA.)

inactive water–$Fe^{(II)}$ prosthetic group to afford the fatty acid hydroperoxide product (13-*S*-linoleic acid hydroperoxide for the major soybean lipoxygenase) and cycle the enzyme back to an active state.

6.2.4.2.7 Net Effects on Enzyme Catalysis

The net effects of bringing various combinations of mechanisms to bear on enzyme catalysis are estimated to deliver as much as 10^{17}–10^{19} in reaction rate enhancement over uncatalyzed reactions [47,96,141]. Most of this enhancement is by transition-state stabilization (reduction of activation energy) and a small contribution may derive from the process of tunneling, particularly in hydrogen transfer steps.

6.2.5 Kinetics of Enzyme Reactions

The mechanisms of enzyme catalysis described above accounts for the chemistry of substrate transformation, but they do little to characterize the kinetics of enzyme reactions (*how fast they go*). Since enzymes are used to hasten reactions in a manner to improve and/or add value to foods, knowledge of how fast enzyme reactions can proceed is a critical factor in deciding if and when an enzyme process should be used. Since enzymes are also selective, knowing *how much more selective* an enzyme is for one substrate over another, or relative to a nonenzymic reaction, may also be a critical factor in governing the choice of using an enzyme process. Rates of any reaction, enzymic or not, depend on intrinsic kinetic factors (related to activation energies; Figures 6.1 and 6.2) as well as on the concentrations of reactants and catalyst (mass action effects). Since concentrations may vary between reaction conditions, it is most valid to compare relative catalytic power on the basis of intrinsic factors such as kinetic constants. If reaction *rate constants* are known for a set of environmental conditions, then *reaction rates can be predicted* for any combination of reactant and catalyst concentrations under those general conditions.

6.2.5.1 Simple Models for Enzyme Reactions [30,113]

Enzymes are fairly unique in the type of kinetics they exhibit. Consider the simplest enzyme reaction, the rapid equilibrium model known as Michaelis–Menten kinetics. Here an enzyme (E) acts upon a single substrate (S) to form a single association complex (ES) (sometimes called the Michaelis complex) that yields a single product (P):

$$\mathrm{E} + \mathrm{S} \underset{k_1}{\overset{k_{-1}}{\rightleftarrows}} \mathrm{ES} \underset{k_{\mathrm{cat}}}{\rightarrow} \mathrm{E} + \mathrm{P} \tag{6.9}$$

The binding of S to E is assumed to represent equilibrium conditions between the association (E + S → ES) and dissociation steps (ES → E + S), each with a respective and characteristic second-order (k_1) and first-order (k_{-1}) rate constant. Biochemical convention is to represent binding equilibria as dissociation processes, and thus, the equilibrium condition for the substrate binding step is expressed as

$$\frac{[\mathrm{E}] \times [\mathrm{S}]}{[\mathrm{ES}]} = \frac{k_{-1}}{k_1} = K_{\mathrm{S}} \quad \text{(the dissociation or affinity constant)} \tag{6.10}$$

Note that a decreasing value of K_{S} indicates that a greater proportion of enzyme exists in the ES form, and there exists greater binding or affinity between E and S. The second stage of the enzyme reaction is the catalytic step of ES → E + P, characterized by the first-order catalytic rate constant, k_{cat}. Thus, the initial rate or velocity (v) of an enzyme reaction can be represented as

$$v = \mathrm{d}\mathrm{P}/\mathrm{d}t = k_{\mathrm{cat}}[\mathrm{ES}] \tag{6.11}$$

and the rate of P formation in this model is viewed as not disturbing the binding equilibria between E and S; hence, the reference to the rapid equilibrium model for enzyme kinetics.

An alternative kinetic approach assumes that the rate of decomposition of ES to form P can influence the proportion or distribution of enzyme between the free E and ES states. To reconcile this, it can be assumed that over a brief period of time a reaction is observed, the [ES] does not change or changes negligibly (this is referred to as the steady-state approach, developed by G. Briggs and J. Haldane). Under this scenario

$$\mathrm{d}[\mathrm{ES}]/\mathrm{d}t \approx 0 \tag{6.12}$$

Thus, the rate of formation of ES is equivalent to rate of disappearance of ES. Since ES formation comes from binding of S with E (the k_1 step) and the disappearance of ES is accounted for by the sum of the processes of ES dissociation (the k_{-1} and k_{cat} steps):

$$k_1[\mathrm{E}] \times [\mathrm{S}] = (k_{-1} + k_{\mathrm{cat}})[\mathrm{ES}] \tag{6.13}$$

This equation can be rearranged as a dissociation process to

$$\frac{[\mathrm{E}] \times [\mathrm{S}]}{[\mathrm{ES}]} = \frac{(k_{-1} + k_{\mathrm{cat}})}{k_1} = K_{\mathrm{M}} \quad \text{(the Michaelis constant)} \tag{6.14}$$

This equation is similar to Equation 6.10, except that it allows for [ES] to be dictated by both the dissociation and catalytic pathways. Also, key to the relationship between K_{S} (Equation 6.10) and K_{M} (Equation 6.14) is the relative magnitude of k_{-1} and k_{cat}. If k_{cat} is a couple of orders of magnitude or so less than k_{-1}, then k_{cat} can be ignored and the distribution of enzyme between E and ES is dictated *only* by the binding equilibrium, rendering K_{M} equivalent to K_{S}. If on the other hand, k_{cat}

is within an order of magnitude or so of k_{-1}, then the predicted binding equilibrium distribution of enzyme between E and ES will never be reached, because the k_{cat} step is sufficiently fast to deplete ES to less than equilibrium levels. Thus, in this case, $K_M \neq K_S$ and K_M does not simply indicate affinity. Enzymes behaving in this manner are considered to conform to steady-state kinetic models. K_M is referred to as a pseudo-dissociation constant for ES, and it has the units of molarity (M), as does S (and K_S). This allows K_M and [S] to be directly compared, since they have the same units, and the utility in this relationship will be shown later. In cases when $k_{cat} \gg k_{-1}$, $k_{cat}/K_M = k_1$, which means the reaction is rate-limited by the association step. Since association rate constants for enzymes are often $\sim 10^7$–10^8 s^{-1} M^{-1}, the existence of steady-state conditions can be diagnosed by the estimated k_{cat}/K_M values, the values being 10^6–10^8 s^{-1} M^{-1} [41,138]. Many oxidation–reduction and isomerizing enzymes exhibit steady-state kinetics, while most (but not all) hydrolytic enzymes do not (thus, for most hydrolytic enzymes $K_M \approx K_S$, and K_M *is* usually a measure of affinity).

6.2.5.2 Rate Expressions for Enzyme Reactions

Enzyme reaction rate expressions can be devised by taking the ratios of two equivalencies, the velocity expression (Equation 6.11) and an expression for the conservation of total enzyme (E_T):

$$\frac{v}{[E_T]} = \frac{k_{cat} \times [ES]}{([E] + [ES])} \tag{6.15}$$

The equation is greatly simplified if enzyme species are expressed only in the form [ES], which can be done be rearranging Equation 6.14 as $[E] = (K_M \times [ES])/[S]$, and substituting for [E] in Equation 6.15. If one considers that the fastest an enzyme reaction can proceed (V_{max}) is when all enzyme is in the ES form, then

$$V_{max} = k_{cat} \times [E_T] \tag{6.16}$$

Equation 6.15 now simplifies to

$$v = \frac{V_{max} \times [S]}{(K_M + [S])} \tag{6.17}$$

This becomes a very powerful relationship in many ways. Since V_{max} and K_M are constants, this equation takes on the form of

$$y = \frac{ax}{(b + x)} \tag{6.18}$$

This equation, where a and b are constants, is defined as a rectangular hyperbola, and simple enzyme kinetics are often referred to as hyperbolic kinetics. Equation 6.17 also helps illustrate how enzyme reaction rates are dependent on substrate, and at low [S], $K_M \gg$ [S], and

$$v = \frac{V_{max} \times [S]}{K_M} \tag{6.19}$$

Thus, when S is at limiting concentrations or at infinite dilution, the rate of the reaction is characterized by the combined constant V_{max}/K_M, the reaction is first order with respect to S, and the enzyme reaction at dilute [S] is depicted as

$$E + S \xrightarrow{V_{max}/K_M} E + P \tag{6.20}$$

This model corresponds to the ability of an enzyme to recognize and then transform a substrate at a dilute state, and this provides for a measure of "catalytic efficiency," which is quantified by the constant V_{max}/K_M (also called the "specificity constant"). Quantitative comparisons of enzyme selectivity toward multiple substrates, based on V_{max}/K_M values, allow inferences to be made as to how the enzyme recognizes substrates (Section 6.2.6). Since V_{max}/K_M are constants, the comparison of selectivity constants is valid at all levels of [S] among competing substrates. At the other extreme, if [S] $\gg K_M$, then Equation 6.17 simplifies to

$$v = V_{max} \tag{6.21}$$

It should be obvious that the reaction rate is zero order with respect to [S], and under this condition all enzyme is "saturated" with substrate, such that the enzyme reaction can be modeled simply as

$$ES \xrightarrow{k_{cat}} E + P \tag{6.22}$$

The importance of this situation is that the reaction rate is dependent only on $[E_T]$ (recall Equation 6.16), and this condition is important to satisfy if one wishes to develop an assay to quantify how much enzyme activity is present, such as the case when enzyme activity is used as indicators of processing efficacy.

There may be cases when enzyme reactions do not conform to conventional Michaelis–Menten, either because the model does not apply, or the ability to fit experimental data to the model is obscured by others factors in play (e.g., S inhibition, endogenous inhibitor in S, multiple enzymes causing the same reaction). These and other complexities may be reconciled by more advanced techniques [30,113]. In any case, the use of terms such as K_M are reserved only for situations where Michaelis–Menten behavior is validated, otherwise terms such as $S_{0.5}$ and $K_{0.5}$ are recommended as analogous terms.

Other kinetic models and relationships applied less frequently to enzyme systems in foods will not be discussed in this chapter. However, they are important to identify and include bisubstrate reactions with a compulsory or random order of addition of substrates and/or products, equilibrium reactions, and allosteric enzymes [30,113].

6.2.5.3 Graphical Analysis of Enzyme Reactions

Between the extreme cases of infinite concentration (saturation) and infinite dilution of S, it is easy to predict enzyme reaction rates if one knows the relative values of V_{max}, K_M, and S; the latter two have units of molarity, such that S can be expressed as multiples of K_M (xK_M). And if v is expressed as a proportion of V_{max} (divide both sides of Equation 6.17 by V_{max}), the enzyme reaction rate expression simplifies to

$$\frac{v}{V_{max}} = \frac{xK_M}{(K_M + xK_M)} \tag{6.23}$$

If one substitutes a series of values $(1, 2, 3, \ldots$ and $0.5, 0.33, 0.2, \ldots)$ for "x" in Equation 6.23, one can construct a typical enzyme kinetic relationship as a function of [S] or $[S]/K_M$, which yields a rectangular hyperbola (Figure 6.9; one asymptote is V_{max}, while the other is at an S/K_M value of -1). This figure shows how the reaction is first order with respect to [S] with a slope of the tangent drawn toward infinite dilution of [S] equivalent to V_{max}/K_M as predicted by Equation 6.19. The reaction approaches zero order as [S] increases and enzyme saturation is approached. Furthermore, such a plot can be constructed after V_{max} and K_M are determined for an enzyme reaction, and there should be good fit between observed and predicted behavior. If not, this means the enzyme does not behave

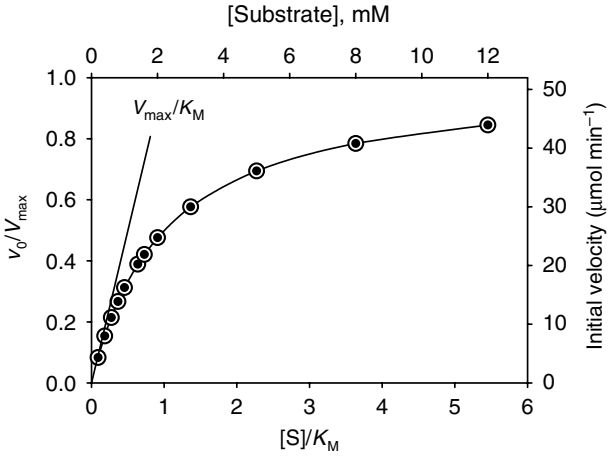


FIGURE 6.9 Michaelis–Menten (hyperbolic) kinetics. Hypothetical enzyme was assumed to have a V_{max} of 52 μmol min^{-1} and a K_M of 2.2 mM. Open symbols represent data plotted on the left ordinate/lower axis; closed symbols represent data plotted on the right ordinate and upper axis. Curved line plot represents nonlinear regression fit.

strictly according to the Michaelis–Menten model, suggesting greater complexity to the nature of the reaction.*

The determination of V_{max} (proportional to k_{cat}) and K_M are important for any enzyme of interest, because it is these two terms that allow one to predict how fast catalysis will take place over a range of conditions of E and S. A particularly useful application of kinetic parameters in food processing derives from the integrated form of the Michaelis–Menten velocity expression

$$V_{max} \times t = K_M \times \ln([S_0]/[S]) + ([S_0] - [S]) \tag{6.24}$$

where S_0 is the initial substrate concentration and S is the substrate concentration at time t. The time required for a desired fractional conversion (X) of substrate [$X = (S_0 - S)/S_0$] is

$$t = SX + K_M \times \ln[1/(1 - X)]/V_{max} \tag{6.25}$$

This relationship can provide a reasonable estimate as to how much enzyme (V_{max} term) must be added to achieve a specified extent of reaction within a specified time period (such as in a processing situation). This equation can only provide rough estimates as there are many reasons why enzyme activity may depart from the predicted course, and they include depletion of coreactant/substrate, product inhibition, progressive enzyme deactivation, change in conditions affecting reaction progress, among others.

The rate constants derived from the Michaelis–Menten equation have other meanings. The first-order constant k_{cat} relates only to the behavior of ES and other similar species (other intermediates plus the enzyme–product complex, EP). Recall that this constant is also called the enzyme turnover number. K_M, the "Michaelis constant," is often referred to as the *apparent dissociation constant*, since this constant may be representative of the behavior of multiple enzyme-bound species (see Figure 6.3 as an example). The "apparent" designation also derives from K_M often being determined

* Many complex enzyme reactions, such as multisubstrate reactions, will exhibit typical hyperbolic kinetics as long as only one substrate is limiting to, or varied for, the reaction, such that it behaves kinetically as a single substrate or "pseudo-first order" reaction.

by experimental data generating v vs. [S] plots and not by the direct determination of composite rate constants (k_1, k_{-1}, and k_{cat}). K_M is the substrate concentration where the enzyme reacts at $\frac{1}{2}V_{max}$, and where enzyme is half-saturated by substrate. K_M is theoretically independent of [E], although anomalous behavior can occur, especially in concentrated and complex enzyme systems. Last, comparing K_M with [S] in a food matrix can be quite revealing. Intermediate metabolites in cellular systems are often present at concentrations in the range of K_M, since this allows for fine reaction control where activity can increase or decrease with a subtle change in [S] [121]. In contrast, if [S] $\gg K_M$ in cellular systems, this implies some barrier to enzyme activity on that substrate must exist (such as physical separation or "compartmentation") for the condition of [S] $\gg K_M$ to persist. While K_M for many enzymes and their substrates is in the range of 10^{-6}–10^{-2} M, some K_M values can be quite high at 40 mM for glucose oxidase toward glucose, 250 mM for xylose (glucose) isomerase toward glucose and 1.1 M for catalase toward H_2O_2 [141]. The apparent second-order rate constant, k_{cat}/K_M (proportional to V_{max}/K_M) relates to properties of the free enzyme (recall Equation 6.20) and is also called the "specificity constant." The magnitude of this constant cannot be greater than any other second-order constant for the enzyme system, and as such represents a minimum value for the association constant (k_1 step in Equation 6.9) for an enzyme–substrate system.

6.2.5.3.1 Critical Features of Enzyme Assays

While understanding that kinetic characterization of enzyme reactions helps guide their use and control in food matrices, it is equally important to understand how to derive such constants with accuracy and confidence. The traditional approach is to collect experimental observations on how reaction velocity (v) varies with [S] (as in Figure 6.9). Reaction progress can be monitored using continuous or discontinuous methods, where P accumulates over time, to yield a collection of reaction rate data (Figure 6.10). One of the most critical issues is to ensure that "linear rates" or "initial velocities" (v_0) are being measured, since the rate expressions developed on the basis of the Michaelis–Menten (and many other kinetic) models are valid only for a specific initial level of substrate [S_0], and not as [S] declines. In practice, this is achieved by allowing for no more than 5–10% of the original [S] to be consumed during the period of observation [28]. This is especially important at low initial [S] ([S_0] $< K_M$) where the reaction rate approaches first order with respect to [S]. Even in this case, one can still estimate the linear rate or v_0 by drawing a tangent and "linearizing" the initial portion of the reaction progress curve (see Figure 6.10). There is less opportunity at [S_0] $\gg K_M$ for the reaction to deviate from linearity since the reaction will remain nearly zero order with respect

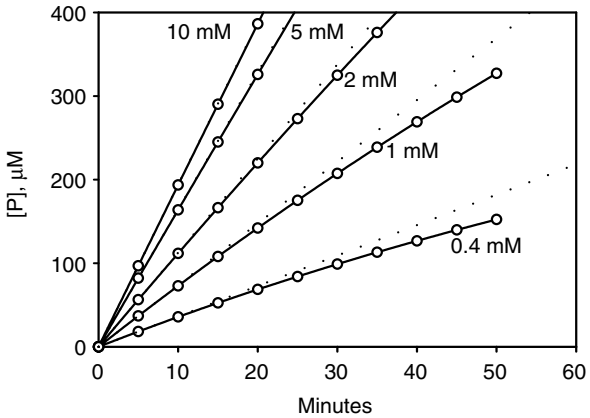


FIGURE 6.10 Progress curves of enzyme reactions as a function of [S]. Reaction progress is based on the hypothetical enzyme parameters in Figure 6.9 legend and appear as solid line and symbol plots. Tangents to the initial velocity or "linear" portion of the curves appear as broken line plots.

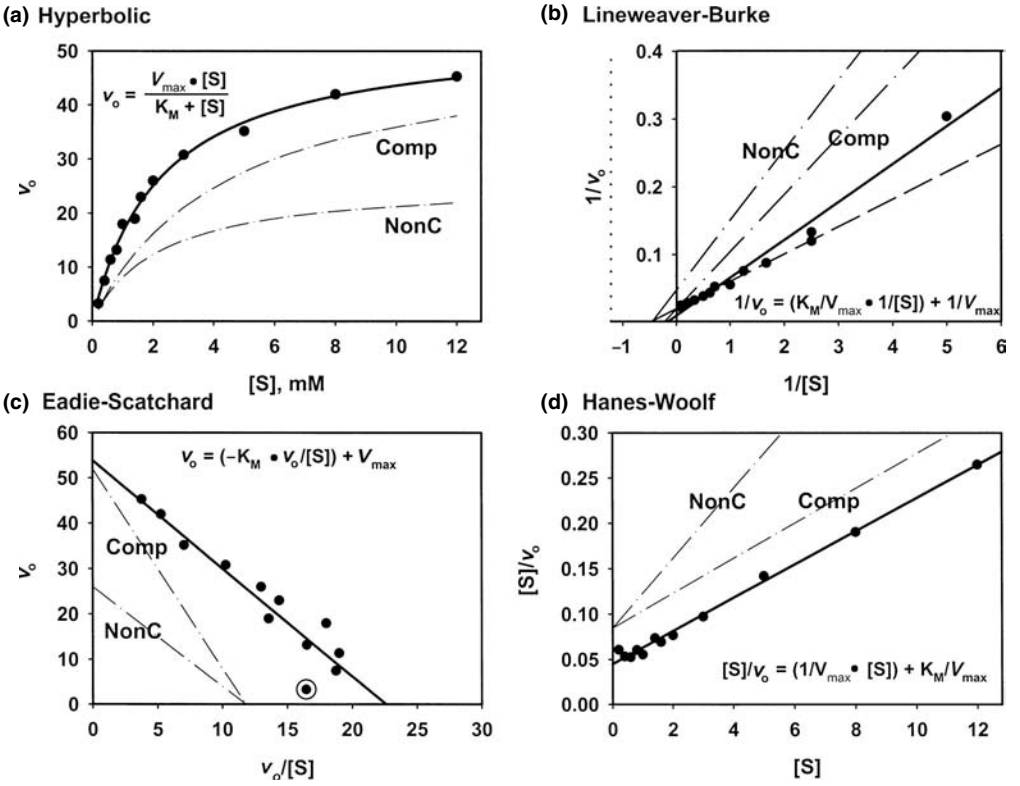


FIGURE 6.11 Hyperbolic and linear transformation plots of enzyme rate data. Hypothetical experimental observations for an enzyme with kinetic parameters approximating those in Figure 6.9 legend appear as best-fit solid line and symbol plots. Equations for all linear plots are expressed in the form of $y = mx + b$. Dot-and-dash broken-line plots are for the types on inhibition modeled in Figure 6.12, and assuming [inhibitor] and K_I values of 0.8 and 0.5 mM, respectively, for both competitive (Comp) and noncompetitive (NonC) inhibition. Broken line (dashed) plot in panel (b) is uninhibited reaction corrected for "outlier" data point observed at lowest [substrate] evaluated; this outlier point is identified in panel (c).

to [S] even after >10% depletion of [S_0]. In addition to the complications of dependence of reaction rates when [S] < K_M, greater *degree of error* is typically encountered when measuring the lower reaction rates within a range of [S], based on the limits of sensitivity of the assay (analytical) method.

6.2.5.3.2 Estimation of K_M and V_{max}

A common way to estimate K_M and V_{max} from experimental rate data is by using any one of three linear transformations of the original Michaelis–Menten rate expression (Equation 6.17, Figure 6.11). Although these transforms take different forms, they are mathematically equivalent and should yield identical results, using accurate data. However, all experimental observations have embedded error, and these errors can differentiate strengths and weaknesses in these alternative linear methods. The most commonly used (and misused) linear transformation is the double reciprocal (Lineweaver–Burke) plot [44,54]. The primary limitation of this plot is that greatest weight is placed on the weakest data points of the set (i.e., the lowest [S] studied are subject to the greatest % error), and the degree of uncertainly (error, along the *y*-axis) is further amplified by the reciprocal nature of the coordinates (Figure 6.11b). Thus, even modest error or uncertainty can greatly influence the placement of the regression line. In all fairness, Lineweaver and Burke recognized that appropriate "weighting" of coordinates should be exercised, but this is largely ignored today. The Hanes–Woolf

plot is opposite to the double reciprocal plot in that it places greatest emphasis (weight) on data points least encumbered with error (at the highest [S] in the set) (Figure 6.11d). However, this also creates graphical bias within the data set toward the [S] $>$ K_M portion of the curve. Last, the Eadie–Scatchard plot places even weight on each data point of the set, but suffers from error (uncertainty) being encountered on both axes, as the dependent variable (v_0) constitutes a factor in each (Figure 6.11c). This linear plot also finds utility in that it allows easier identification of "outlier" data points than the other plots (the point at the lowest v_0 stands out).

Regardless of which plot is used, the data set must include observations that comprise a good balance of [S] above, below, and near K_M [30,113]. This prevents the data set from being too biased toward the first- or zero-order region of the hyperbolic curve (Figure 6.9). More precisely, it is the response of reaction rate to the region where [S]/K_M ranges from 0.3 to 3 (or 0.5 to 5) that is most important and serves to define the curvature of the plot and how rate depends on [S]. Linear transformations are not the only way to estimate kinetic constants of enzyme reactions. Many even rudimentary graphics software programs today allow experimental data to be fitted to a rectangular hyperbola, a specific nonlinear regression fit (Equation 6.17; Figures 6.9 and 6.11a) to obtain estimates of K_M and V_{max} values directly from the original (and nontransformed) data set. This curve also allows for reasonable visual estimates of K_M and V_{max}, and how well actual data conform to the fitted curve.

Linear plots also find utility in characterizing action of inhibitors (I) of enzyme reactions (Figure 6.11, broken-line plots). The two common types of inhibition are competitive and noncompetitive (Figure 6.12). Competitive inhibitors have structures that resemble those of substrates and interfere with S binding at the active site, making the enzyme reaction behave as having an elevated K_S or K_M value (without affecting the k_{cat} step or V_{max} value). On the other hand, noncompetitive inhibitors do not interfere with S binding (have no impact of the K_S or K_M value), but effectively "poison" the enzyme by reducing the V_{max} by a proportion equivalent to the amount of enzyme bound to inhibitor ([EI] + [ESI]) at a given [I] and respective inhibitor dissociation constant (K_I) in the system. The effect of a competitive inhibitor can be ameliorated by adding excess [S] to "out-compete" the inhibitor and pulling the reaction equilibria toward ES and ES $\rightarrow$ E + P. In contrast, for the noncompetitive inhibitor, this does not occur because inhibitor can bind to either E or ES, and thus the amount of [EI + ESI] is not affected by [S] at a given [I]. Close inspection of the corresponding slopes and intercepts of the lines representing the two types of inhibition in the linear plots (Figure 6.11b–d) reveals that V_{max} remains constant while K_M increases for competitive

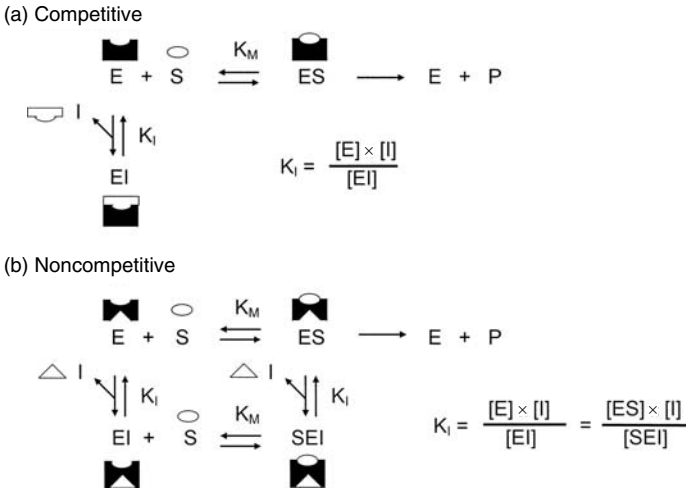


FIGURE 6.12 Model for simple (a) competitive and (b) non-competitive inhibition of enzyme reactions.

inhibition and V_{max} decreases and K_M remains constant for noncompetitive inhibition, relative to reactions without inhibitor. Equations for K_I values for these types of inhibition appear in Figure 6.12, and K_M and V_{max} values are modified by the factor of $(1 + [I]/K_I)$ as appropriate [30,113].

Other and lesser common types of inhibition include suicide inhibitors (substrates) that bind to the active site and are transformed by the enzyme to a derivative that reacts with and deactivates the enzyme, and *un*competitive inhibitors that only bind to the ES species and inhibit enzyme action. Reports of *un*competitive inhibition should be treated with great skepticism, since there are only a few documented cases of this type of behavior [29].

6.2.6 Specificity and Selectivity of Enzyme Action [41]

Although the terms specificity and selectivity are often used interchangeably, these terms relate to the *discriminatory power* of enzyme action. Enzymes can discriminate between competing substrates on the basis of differential binding affinities and facility of catalysis. An enzyme can be *specific* if it reacts only with substrates that have a certain type of chemical bond (e.g., peptide, ester, glycoside) or group (e.g., aldohexose, alcohol, pentadiene), or an enzyme may exhibit (near-)absolute specificity, where a single chemical reaction is catalyzed for a defined substrate(s). In addition, enzymes may also exhibit product specificity and stereochemical specificity. Thus, one can consider *specificity* as denoting the general and/or *exclusive* nature of the type of enzyme reaction catalyzed. The term *selectivity* refers to the relative preference of reactivity of an enzyme toward similar, competing substrates, indexed as V_{max}/K_M (Section 6.2.5). For the casual reader, it is acceptable to use the terms specificity and selectivity interchangeably.

6.2.6.1 Specificity Patterns of Selected Food Enzymes

6.2.6.1.1 Proteolytic Enzymes

Some of the earliest (and considered classic) work on the role of noncatalytic sites of enzymes involved in S recognition involved papain (EC 3.4.22.2), the cysteine protease from papaya latex with commercial application as a meat tenderizing agent. Using a series of synthetic peptide substrates, different sites of the enzyme and substrates were "mapped" [109,110], and the basis of enzyme selectivity was inferred from the relative reactivity of members of this substrate series (Figure 6.13 [109,110]). The formalism developed is now applied to all protease–peptide reactions. The scissile bond of the peptide substrates is designated as that linking residues P_1 and P'_1, while other substrate amino acid residues are sequentially designated $P_2, P_3, \ldots, P_i$ toward the N-terminus, and $P'_2, P'_3, \ldots, P'_i$ toward the C-terminus. The corresponding sites of papain that interact with the substrate subsites are designated S and S′ with the same numeric codes as the corresponding substrate residues. While the P series of substrate residues corresponds to a single amino acid, one or multiple amino acid residues may share and comprise the same S_x "space" to collectively interact with a corresponding substrate residue. The selectivity data used to "map" the important residues of papain also appear in Figure 6.13.

While papain is considered to have broad selectivity in hydrolyzing peptide bonds, this study showed a clear preference for substrates with PHE (aromatic/nonpolar residue) at the P_2 site of the substrate (other substrates examined are not included in the figure). Consequently, although PHE is not part of the peptide bond hydrolyzed, the enzyme exhibits a preference in recognizing PHE at the S_2 site and this dictates which peptide bond is brought into register as the scissile bond. It is inferred that the S_2 subsite "space" in papain is occupied by similarly hydrophobic residue(s), and that the interaction between the P_2 and S_2 residues makes a major contribution to papain selectivity for peptide bond hydrolysis. The active site of papain comprises a deep cleft, with the catalytic residues CYS_{25} and HIS_{159} on opposite sides of the cleft [116]. Up to seven nonpolar residues from both sides of the cleft are implicated as comprising the S_2 space of papain. By comparison, serine proteases primarily exhibit substrate selectivity through interactions at (sub)sites S_1/P_1, and the

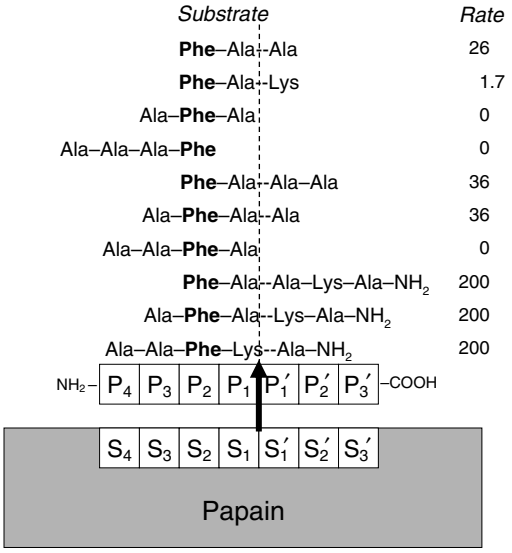


FIGURE 6.13 Substrate "mapping" of the active site of papain by kinetic analysis using peptide substrates. (Data was selected and figure adapted from Schechter, I. and Berger, A. (1967). *Biochem. Biophys. Res. Comm.* 27:157–162 and Schechter, I. and Berger, A. (1968). *Biochem. Biophys. Res. Comm.* 32:898–912.) Reaction rates are normalized by the author since reactivity of substrates was determined by end-point analysis after different incubation times (initial velocities were not measured). Arrow and dashed line indicate the register for the scissile peptide bond.

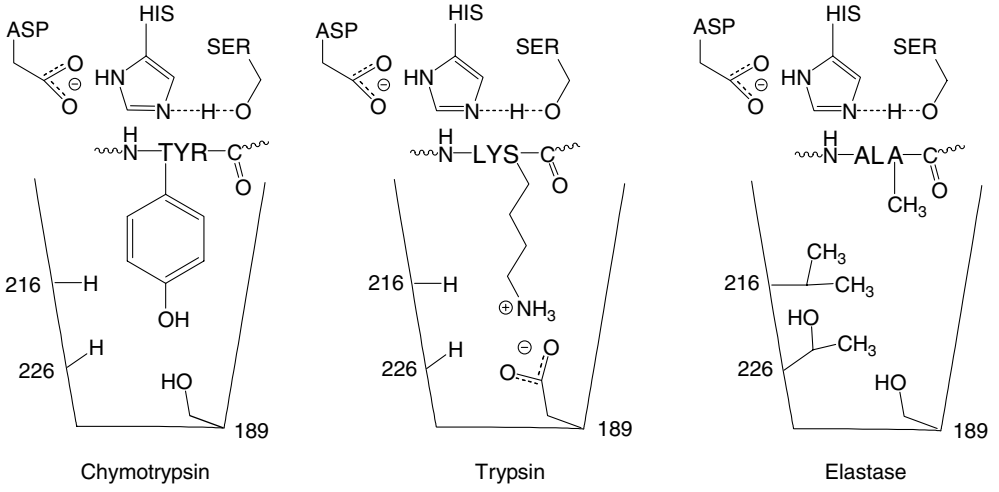


FIGURE 6.14 Substrate binding pockets for serine proteases. Preferred P_1 amino acid side chain is shown in binding pocket with other amino acid side chains of enzyme at sites 216, 226, and 189. (Adapted from Fersht, A. (1985). *Enzyme Structure and Mechanism*, 2nd edn., W. H. Freeman & Company, New York and Whitaker, J.R. (1994). *Principles of Enzymology for the Food Sciences*, 2nd edn., Marcel Dekker, New York.)

critical amino acid residues and the resulting bond selectivity for trypsin, chymotrypsin, and elastase are conferred largely by steric and electrostatic factors as shown in Figure 6.14 [41,140].

Perhaps, no enzyme is better known for its reaction selectivity than is the acid protease chymosin (EC 3.4.23.4, also called rennin), used exclusively for cheese making. The crude enzyme preparation,

TABLE 6.4
Enzyme–Substrate Interactions Involved in Chymosin Selectivity for the PHE–MET Bond of κ-Casein

	Peptide			k_{cat}	K_M	k_{cat}/K_M
κ-Casein	100	105 ↓ 106	110	(s^{-1})	(mM)	(s^{-1} mM^{-1})
Ref	His–Pro–His–Pro–His–Leu	Ser–Phe–Met–Ala	Ile–Pro–Pro–Lys–Lys			
a		Ser–Phe–Met–Ala	Ile–OMe	0.33	8.5	0.04
b		Leu–Ser–Phe–Met–Ala	–OMe	0.58	6.9	0.08
c		Leu–Ser–Phe–Met–Ala	Ile–OMe	18.3	0.85	21.6
d		Leu–Ser–Phe–Met–Ala	Ile–Pro–OMe	38.1	0.69	55.2
e		Leu–Ser–Phe–Met–Ala	Ile–Pro–Pro–OMe	43.3	0.41	105
f		Leu–Ser–Phe–Met–Ala	Ile–Pro–Pro–Lys–OH	33.6	0.43	78.3
g		Leu–Ser–Phe–Met–Ala	Ile–Pro–Pro–Lys–Lys–OH	29.0	0.43	66.9
h	His–Pro–His–Pro–His–Leu	Ser–Phe–Met–Ala	Ile–Pro–Pro–Lys–OH	66.2	0.026	2510
i	His–Pro–His–Pro–His–Leu	Ser–Phe–Met–Ala	Ile–Pro–Pro–Lys–Lys–OH	61.9	0.028	2210

Source: Visser, S. (1981). *Neth. Milk Dairy J.* 35:65–88.

called "rennet" and obtained from the stomach of young calves, is highly selective for hydrolyzing the PHE_{105}–MET_{106} bond of κ-casein during the initial milk-clotting phase of cheese making. Kinetic studies of chymosin action on synthetic peptides that modeled portions of the κ-casein substrate revealed factors responsible for its selectivity (Table 6.4 [134]). First, chymosin was found to be a size-selective endopeptidase requiring at least a pentapeptide for activity where neither the PHE or MET could be the terminal residue (data not shown in Table 6.4). Thus, reactivity with peptide fragments (a) and (b) represents a reference or basal level of chymosin activity on the PHE–MET bond in minimally sized peptides. Peptide extension toward the C-terminus of κ-casein (substrates c through g) enhances reaction selectivity (k_{cat}/K_M) toward the PHE–MET bond by 2–3 orders of magnitude over substrate (b), with greater impact on elevating k_{cat} than reducing K_M, although both parameters are affected. This demonstrates the important role that ILE–PRO–$PRO_{108–110}$ residues have on substrate recognition and especially stabilizing the transition state, with the rigidity of the PRO residues playing a pivotal role, perhaps imposing strain/distortion. For the complete κ-casein substrate, PRO may help expose the scissile bond to protease (Chapter 15). Likewise, extending the peptide substrate toward the N-terminus (substrates (h) and (i)) further increases selectivity by two orders of magnitude. This is almost exclusively realized by enhanced affinity (binding) of substrate to the enzyme, as K_M decreases while there is little change in k_{cat}. The positively charged cluster of $HIS_{98,100,102}$ residues at reaction pH helps "freeze" substrate at the active site by coordinating with corresponding electronegative groups on the enzyme at subsites S_8–S_6–S_4, providing for electrostatic attraction. This example demonstrates how substrate structure can enhance reaction selectivity through long-range interactions with enzyme, in this case enhancing selectivity (k_{cat}/K_M) by ~5 orders of magnitude toward the scissile bond. This example also explains why it has been challenging to identify and use "microbial rennets" (chymosin substitutes) for cheese making as these alternative proteases usually have lesser milk-clotting:proteolytic activity ratios (0.10–0.52) than chymosin (1.4), and this leads to continued breakdown of the curd (compromising of textural quality) and undesirable bitterness as the cheese ages [70].

6.2.6.1.2 Glycosyl Hydrolases (Glycosidases) [116,141,147]

Glycosyl hydrolases act on glycosidic bonds on di-, oligo-, and polysaccharides. The nature and extent of enzyme–substrate recognition and subsite mapping has been well studied among this group

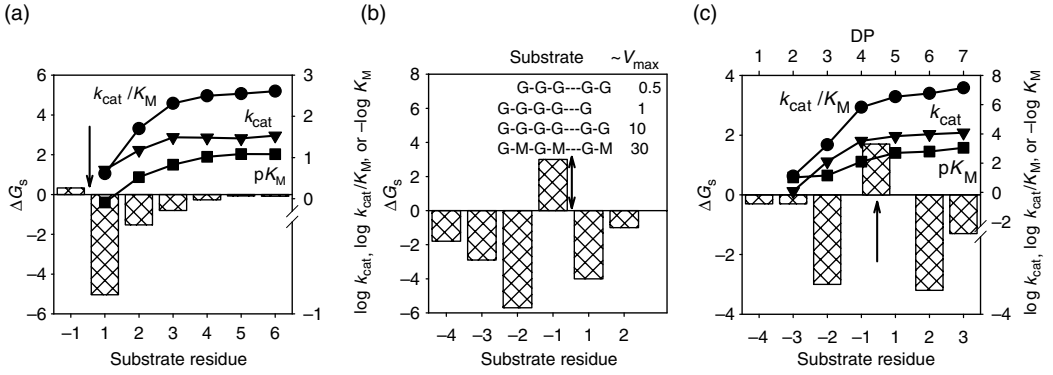


FIGURE 6.15 Substrate subsite mapping of glycosyl hydrolases by kinetic analysis. Activity was analyzed for a series of α-1,4-linked glucose oligomers from 1 to 7 units for (a) glucoamylase and (c) α-amylase. Maltose is the smallest substrate for both enzymes but glucose binding occurs for glucoamylase. For glucoamylase, kinetic constants coincide with substrate length increasing from -1 to n; for α-amylase, kinetic constants coincide with the DP of the oligomers; for (b) lysozyme, kinetic constant is for the model substrates where G = *N*-acetylglucosamine and M = *N*-acetylmuramic acid. Estimates of ΔG_S coincide with each subsite, and arrows indicate scissile bond. (Data obtained from Chipman, D.M. and Sharon, N. (1969). *Science* 165:454–465; Meagher, M.M., et al. (1989). *Biotechnol. Bioeng.* 34:681–688; and Nitta, Y., et al. (1971). *J. Biochem.* 69:567–576.)

of enzymes. Examples include (1) glucoamylase, an exo-acting hydrolase releasing single glucose units from the nonreducing end of linear α,1→4 linked maltooligosaccharides; (2) lysozyme, an endo-acting hydrolase recognizing a repeating α,1→4 linked heterodimer of (*N*-acetylglucosamine [NAG] → *N*-acetylmuramic acid $[NAM]_n$); and (3) α-amylase, an endo-acting hydrolase that randomly cleaves linear α, 1→4 linked $(glucose)_n$ segments in starch (Figure 6.15 [25,74,80]). Analogous to active site mapping of proteases, glycosyl hydrolase substrate binding subsites are mapped as $(-n, \ldots, -2, -1, +1, +2, \ldots, +n)$ [34]. Hydrolysis occurs at the glycosidic bond of the residue furnishing the carbonyl group at subsite -1 and the alcohol group at subsite $+1$. Enzyme–substrate interaction at one or both of these subsites may contribute to an unfavorable free energy change of association ($+\Delta G_S$). This should be expected since the substrate bonds to be transformed need to be elevated to a transition state. Rather, interaction at subsites surrounding the transformed residue(s) contributes to the favorable (negative) ΔG_S of binding, and this binding energy may be used to facilitate catalysis. The extent of enzyme–substrate subsite interaction is "mapped" or confined to where further extending the length of the substrate toward $+n$ or $-n$ subsites has no impact on catalytic parameters. In the specific case of glucoamylase (Figure 6.15a), the $+1$ to $+3$ sites particularly enhance both binding and catalysis, whereas other sites serve to enhance binding and have little effect on catalysis.

For lysozyme (Figure 6.15b), interactions with residues at subsites -2 and $+1$ are similarly pivotal in enhancing reactivity, but even interactions at the more remote subsites of -4 and $+2$ have considerable effect on catalysis [41,138,147]. H-bonding is a primary factor in enzyme–substrate recognition, especially between substrate residues $-4/-3$ and ASP_{101}. Substrate structure is also important as the bulkier NAM residue is preferred as the -1 subsite; the lactyl moieties of NAM are sterically hindered from occupying enzyme binding subsites -4, -2, and $+1$. For α-amylase (Figure 6.15c), the residues immediately adjacent ($-2/+2$) to the scissile maltose unit ($-1/+1$) provide for greatest $-\Delta G_S$ for binding and acceleration of catalysis. Further degree of polymerization (DP) continues to enhance binding (K_M) more than catalysis (k_{cat}). In all three examples, remote enzyme–substrate interactions provide the energy required to stabilize the transition state at the active site.

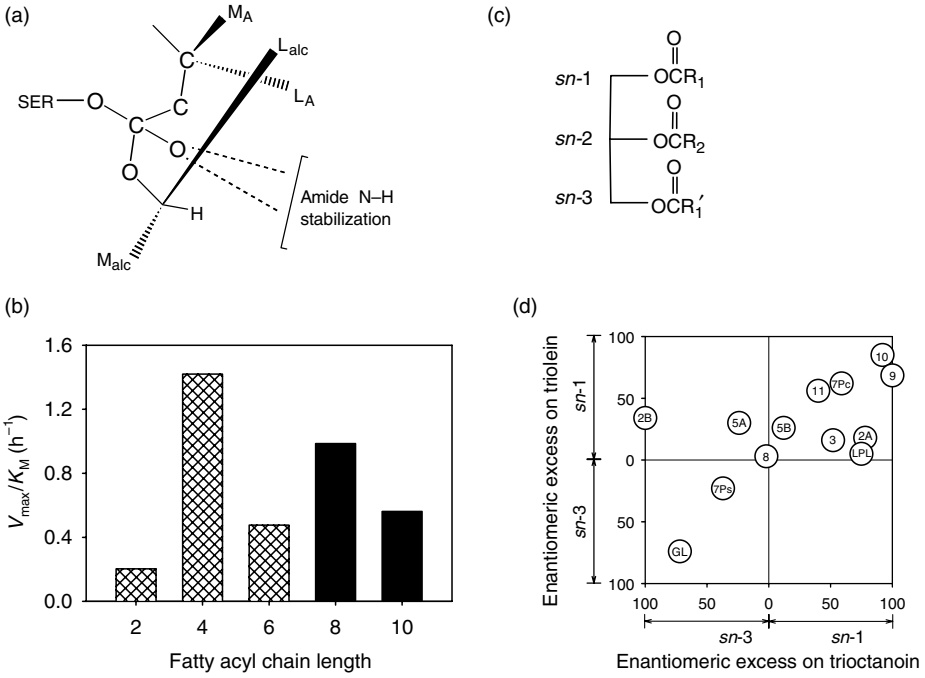


FIGURE 6.16 Features of substrate selectivity of lipases. Panel (a) represents substrate binding orientation and panel (c) represents stereospecific numbering of glycerol backbone. Different shading of bars in panel (b) denotes different stereobias in the reaction among *rac*-α-hydroxylated fatty acid substrates. Numeric coding for lipases in panel (d) appears in Table 6.8, where an accompanying upper case letter refers to an enzyme isoform; LPL = milk lipoprotein lipase; GL = human gastric lipase; 7Ps = *Penicillium simplicissimum* lipase; 7Pc = *P. camembertii* lipase. (Data and Figures adapted from Kazlauskas, R.J. (1994). *Trends Biotechnol.* 12:464–472; Parida, S. and Dordick, J.S. (1993). *J. Org. Chem.* 58:3238–3244; and Rogalska, E., et al. (1993). *Chirality* 5:24–30.)

6.2.6.1.3 Lipid-Transforming Enzymes

With lipases, binding sites exist for both the acyl and alcohol moieties of the ester to be hydrolyzed, and each site possesses two subsites (Figure 6.16a) [59]. These sites are lined with hydrophobic residues and selectivity is largely conferred by volume of the binding pockets. For example, the sizes of the large (L_A) and medium (M_A) acyl subsites of *Candida rugosa* lipase are closely aligned with the respective sizes of the C8 and C4 *n*-acyl groups (Figure 6.16b; [86]), giving rise to the marked preference in reactivity for these acyl groups (but not the closely related C6 *n*-acyl group!). Many lipases exhibit multiple optima for fatty acyl chain length [2,69,99]. The alcohol group of the ester substrate binds at a site exposed to solvent comprising subsites to host the large (L_{alc}) and medium (M_{alc}) constitutive groups of the alcohol moiety (and leaving group; Figure 6.16a). At least three amino acid residues of lipases (adjacent to the catalytic SER/HIS residues and oxyanion-stabilizing amide groups) interact with the M_{alc} group to confer selectivity toward the alcohol group [59]. Other features of substrate (triacylglycerol) binding sites of lipases, including accessibility, volume, and topography, confer regioselectivity toward ester groups (Figure 6.16c, as *sn*-1,3-regiospecific or nonspecific [103]), as well as fatty acid selectivity (e.g., saturated vs. unsaturated) [2,69,99]. The relative contribution of all these selectivity factors toward acyl and alcohol groups governs stereospecificity (almost all mixed triacylglycerols are chiral), and a survey using two model substrates (triolein and trioctanoin) shows the range of stereoselectivity among lipases and how this can be influenced by substrate structure (Figure 6.16d).

A broad scope of factors confers selectivity of lipoxygenases, which react exclusively with the 1,4-pentadiene group of polyunsaturated fatty acids, represented by linoleic acid ($18{:}2_{9c,12c}$).

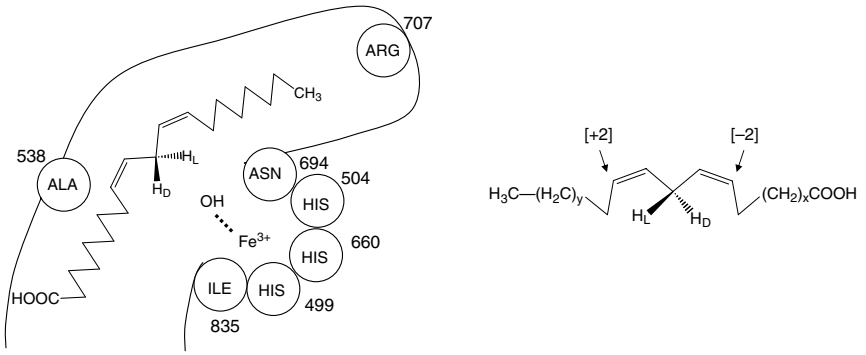


FIGURE 6.17 Active site and positional (stereo)selectivity of lipoxygenase. (Adapted from Boyington, J.C., et al. (1993). *Science* 260:1482–1486; Coffa, G. and Brash, A.R. (2004). *Proc. Natl Acad. Sci. (USA)* 101:15579–15584; Kuhn, H., et al. (1985). *Biochim. Biophys. Acta* 830:25–29; and Prigge, S.T., et al. (1997). *Biochimie* 79:629–636.)

Positional selectivity (regioselectivity) toward oxygenating arachidonic acid ($20{:}4_{5c,8c,11c,14c}$) has emerged as one basis for classifying lipoxygenases (as 5-LOX, 8-LOX, 9-LOX, 11-LOX, 12-LOX, 15-LOX). Lipoxygenases possess two large cavities providing access to the active site. One long, funnel-shaped cavity is lined with hydrophobic residues and serves to provide O_2 access to the active site [96]. The other is also lined with neutral and hydrophobic residues, is rather narrow and bends to form a "boot"-shaped pocket near the active center, and hosts the fatty acid substrate (Figure 6.17 [13,27,62,96]). Lipoxygenases are selective for oxygenating the carbon of the pentadiene at positions [−2] or [+2] from the methylenic carbon (site of H abstraction), relative to the carboxylic acid terminus [62]. This reflects a basic difference in lipoxygenase *product* specificity in how it "counts carbons" based on whether the preferred orientation of substrate binding is carboxylate ([−2] type) or methyl terminus ([+2] type) first entering the binding pocket.

The site of oxygenation also depends on which of possibly multiple 1,4-pentadiene systems ($18{:}3_{9c,12c,15c}$ has two, $20{:}4_{5c,8c,11c,14c}$ has three) is bought in register with the active site iron, and this is partially dependent on the size of the fatty acid binding pocket. Larger binding pockets accommodate longer portions of the fatty acid substrate and shift positional selectivity toward the carboxyl end (such as 5-LOX) for fatty acids inserting methyl group first. The size of the fatty acid binding pocket is also conferred by the size of the amino acid R-groups lining the pocket (in the area of ARG_{707} in Figure 6.17), similar to serine proteases (Figure 6.14). Finally, the product stereospecificity of lipoxygenases in yielding the *S*- or *R*-hydroperoxy fatty acid is related to a single amino acid residue in the enzyme (residue 538 in soybean LOX-isoform 1) being ALA (R-group = CH_3) or GLY (R-group = H), respectively [27]. ALA_{538} sterically obstructs O_2 addition to the proximal (pro-*R*, C-9) site, and confers the 13*S* stereoselectivity, whereas GLY_{538} permits oxygenation at the proximal site, yielding the 9*R* hydroperoxy products (Figure 6.17). This feature applies to all lipoxygenase structures analyzed to date.

Lipoxygenase reaction selectivity also depends on whether the fatty acid is esterified and in what aggregated form (micelles, detergent complexes, or in salt form) and pH (which dictates degree of ionization of the carboxyl group). The pH effect on product selectivity is often explained on the basis of a substrate orientation factor [141]. Soybean LOX-1 exhibits product selectivity at optimum pH ∼9 in that the 13-hydroperoxy-octadienoate is preferred over the 9-hydroperoxy-octadienoate by ∼10:1, while at pH ∼7, the two products are formed in nearly equal proportions. At pH 9, the ionized carboxylate confers positioning of linoleate as shown in Figure 6.17, whereas at pH 7 the protonated linoleic acid may bind in the "inverse" orientation of carboxyl group first, placing the C-9 group in register for the addition of oxygen. This example shows how substrate structure may also influence reaction selectivity.

6.2.6.2 Nomenclature and Classification of Enzymes

Since "trivial" names are often insufficient to represent the precise nature of an enzyme reaction, enzymes are systematically named and catalogued according to rules of nomenclature as defined by the Enzyme Commission (EC) of the International Union of Biochemists and Molecular Biologists (IUBMB). Although trivial names are still used in referring to enzymes, the assignment of an "EC" number removes ambiguity about the specific reaction being described. The EC number comprises four integers, each representing some feature of the enzyme reaction (Table 6.5). The first number describes the general class of the reaction. Hydrolases (class 3) are the most important class of enzymes in food, followed by oxidoreductases (class 1). Trivial names for group transferases (class 2) sometimes include the term "synthase," which does not seem very distinct from the term "synthetase," the latter which is reserved for ligases (class 6), the truly synthetic or bond-forming enzymes. Lyases (class 4) are enzymes that break bonds through nonhydrolytic processes, and trivial names for enzymes that cause reverse "lyase" reactions may include "synthase" and "hydratase." Isomerases (class 5) cause intramolecular rearrangement of atoms. The second and third digits go on to further identify the reaction and the substrate(s) and/or bond(s) transformed. Enzyme reactions lacking in sufficient definition have the third digit assigned as "99." The last digit comprises a "bookkeeping" function to differentiate enzymes sharing the same first two digits, while also providing an additional feature of the reaction to distinguish it from all other enzymes known. Several EC numbers have already been identified in earlier portions of this chapter with early or first mention of specific enzymes.

6.3 USES OF EXOGENOUS ENZYMES IN FOODS [3,48,127,142]

6.3.1 General Considerations

The decision of when to employ an enzyme process is based on several considerations [16,88]. Enzymes are favored when (1) mild conditions are permitted to maintain positive attributes of the food, (2) potential by-products of a chemical process are unacceptable, (3) a chemical process is difficult to control, (4) the "natural" designation is to be retained, (5) the food or ingredient is of premium value, (6) a traditional chemical process needs to be replaced or expanded, or (7) reaction specificity is required. Relative cost-benefit is also a critical factor. Some enzymes can be used as "immobilized" preparations, where they remain active while fixed or bound to inert matrices or particles. This allows the enzyme to be packed in a column/bioreactor through which substrate is perfused, or recovered after batch-reaction with substrate by filtering or settling, such that the enzyme can be used repeatedly until it loses activity beyond an acceptable level. In this manner, enzyme costs are proportionally reduced.

Categorical uses of exogenous enzymes include the production of food ingredients and commodities, such as corn syrups, glucose, high fructose corn syrup, invert sugar and other sweeteners, protein hydrolysates, and structured lipids; modification of components within a food matrix, such as beer stabilization, milk clotting (cheese making), meat tenderization, citrus juice debittering and crumb softening; process improvement, such as cheese ripening, juice extraction, juice/wine clarification, fruit and oil seed extraction, beverage (beer/wine) filtration, faster dough mixing, baked product leavening and stabilization; process control, such as online biosensors; and component analysis. Important uses of exogenous enzymes will be presented on the basis of the nature of the food component undergoing transformation.

6.3.2 Carbohydrate-Transforming Enzymes [116,141,147]

Most enzymes used commercially to act on food carbohydrates are hydrolytic and are collectively referred to as glycosyl hydrolases or glycosidases. Some of these enzymes may catalyze glycosyl

TABLE 6.5
Systematic Nomenclature Rules and Guidelines for Classification of Enzymes

1st #, Class of Enzyme (Reaction Type)	2nd #, Subclass Substrate, Donor, Bond (Examples)	3rd #, Sub-Subclass Other Distinguishing Group, Substrate, Acceptor, Trait (Examples)	4th #, Bookkeeping Serial Number to Differentiate Enzymes That Share Same First Three Numbers (Examples, Common Names)	Format for Systematic Naming
1. Oxidoreductase (oxidation–reduction)	Group in donor oxidized	Acceptor reduced		Donor:acceptor oxidoreductase
	1. CH—OH group	1. NAD(P)	1.1.1.1 Alcohol dehydrogenase	
	10. Diphenol (or related)	3. O_2	1.10.3.1 Catechol (diphenol) oxidase	
	13. Single donor, O_2	11. Two O atoms incorporated	1.13.11.12 Lipoxygenase	
	14. Paired donors, O_2	18. One O atom incorporated	1.14.18.1 Monophenol monooxygenase	
2. Transferase (group transfer)	Group transferred	Group further delineated		Donor:acceptor grouptransferase
	3. Acyl group	1. Other than amino group	2.3.1.175 Alcohol acyltransferase	
	4. Glycosyl group	2. Amino group	2.3.2.13 Transglutaminase	
		1. Hexosyl group	2.4.1.19 Cyclodextrin glycosyltransferase	
3. Hydrolase (hydrolysis)	Bond hydrolyzed	Substrate class		Hydrolase
	1. Esters	1. Carboxylic ester	3.1.1.3 Lipase	
	2. Glycosidase	1. *O*- or *S*-glycosyl	3.2.1.147 Myrosinase (thioglucosidase)	
	4. Peptide	24. Metallopeptidase	3.4.24.27 Thermolysin	
4. Lyase (elimination)	Bond cleaved	Group eliminated		Substrate group lyase
	1. C—C	2. Aldehyde lyase	4.1.2.32 TMNO aldolase	
	2. C—O	2. Act on polysaccharides	4.2.2.10 Pectin lyase	
	4. C—S	1. (None, only 23 enzymes)	4.4.1.4 Alliin lyase	
5. Isomerase (isomerization)	Type of reaction	Substrate, position, chirality		Racemase, epimerase, isomerase, mutase
	2. *cis–trans* isomerase	1. (None, only 10 enzymes)	5.2.1.5 Linoleate isomerase	
	3. Intramolecular redox	1. Aldose–ketose interconverting	5.3.1.5 Xylose isomerase	
6. Ligase (bond-formation)	Bond synthesized	Substrate, cosubstrate(s)		X—Y ligase (synthetase)
	4. C—C	2. Acid–amino acid (peptide)	6.3.2.3 Glutathione synthetase	

Source: IUBMB, http://www.chem.qmul.ac.uk/iubmb/

group transfers and/or reverse hydrolytic reactions in food processes where substrate levels are often high (30–40% solids) due to mass action effects. This group of enzymes accounts for about half of enzyme use (cost basis) as processing aids in the food industry, primarily for the production of sweetener and bulking/thickening agents (dextrins) from starch, and for carbohydrate modification in baking applications. Specialty applications for various glycosidases continue to emerge.

Some general properties of this group of enzymes are well established, derived from the structural and sequence analysis of members of over 60 sequence-based families of glycosidases. Glycosyl hydrolases act on glycosidic bonds, and this group of enzymes share many structural and catalytic properties. Many glycosidases are multidomain proteins, where one portion of the protein functions as the catalytic unit and other domains have alternative functions, one being to bind extended polysaccharide substrates. Glycosidase active sites contain dual carboxyl/carboxylate residues (ASP/GLU) similar to what was shown for the mechanism of lysozyme (Equation 6.8). Mechanistically, this group of enzymes functions by either general acid–base catalysis and/or nucleophilic catalysis (with assistance from electrostatic and strain/distortion effects). In all cases, an acidic residue donates an H^+ to the glycosidic O atom to yield an oxocarbenium ion as the transition state (Figure 6.18 [116]). The carboxylate residue either deprotonates and activates water to yield the nucleophilic –OH to complete the hydrolysis, or the carboxylate can act directly as a nucleophile and form a covalent intermediate; in both cases the alcohol residue is released as the leaving group.

Glycosidases can be categorized either as "retaining" or "inverting" types, based on the fate of the anomeric configuration (α or β) of the hydrolyzed glycosidic bond (Figure 6.18). Inverting types have a larger distance between the catalytic acid residues (~9.5 Å), allowing the activated water

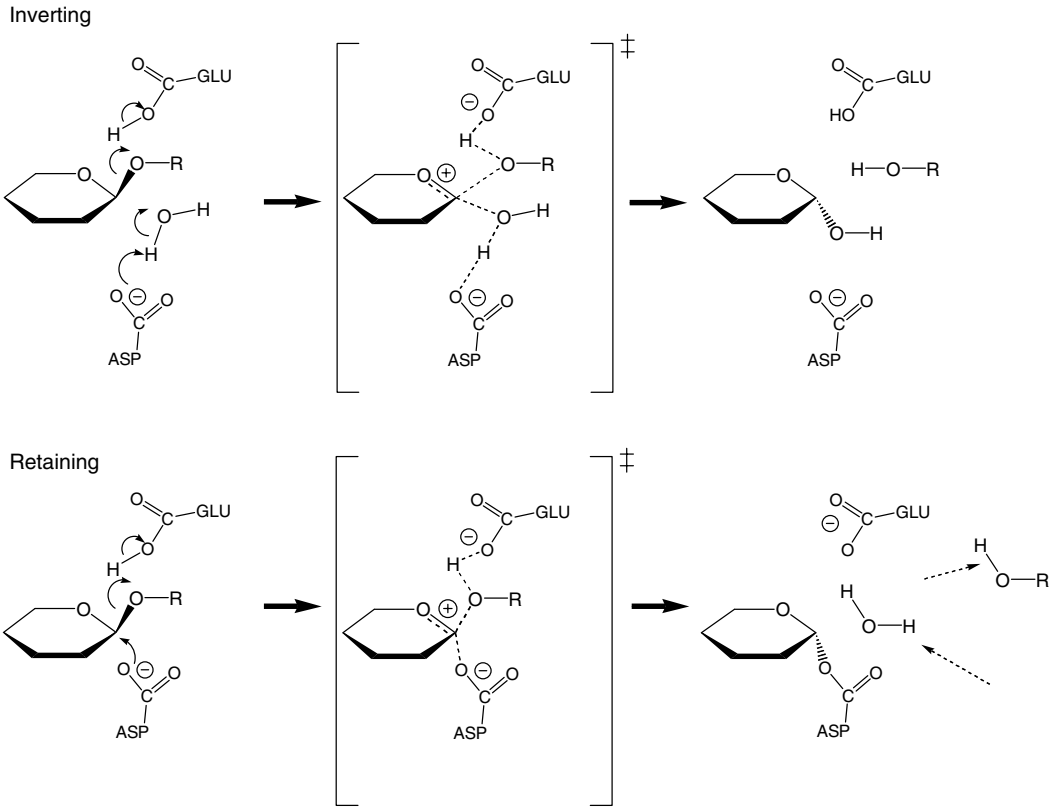


FIGURE 6.18 Mechanistic diversity among glycosyl hydrolases. (Adapted from Sinnott, M. (Ed.) (1998). *Comprehensive Biological Catalysis. A Mechanistic Reference*, Vol. I, Academic Press, San Diego, CA.)

molecule (nucleophile) access to the alternative anomeric site relative to the site of ROH release from the glycosidic bond. Retaining types have shorter spacing between catalytic residues (~5.5 Å) such that water enters the active site only *after* the released alcohol group departs the active site (referred to as a double-displacement reaction). In the retaining reaction mechanism, the glycosyl–enzyme covalent intermediate formed with the carboxylate residue serves to direct water (rendered nucleophilic by the general base residue removing H^+) to the same anomeric position that the ROH leaving group formerly occupied, and thus, the anomeric configuration is "retained." Only retaining glycosidases catalyze both hydrolysis and glycosyl-transfer reactions, whereas inverting types only catalyze hydrolysis reactions. Another general distinction among glycosidases is whether they are "endo" or "exo" acting. Exo-acting types bind the terminal (mostly, but not always, the nonreducing end) portion of the substrate in register as the scissile bond at the active site, whereas endo-acting types randomly attack interior sites of the substrate. Trivial naming of glycosidases as "α" and "β" (as in amylases and glucosidases) recognizes the anomeric configuration of the liberated reducing group as being axial and equatorial, respectively. A summary of the types and classification of glycosidases of most importance in foods is provided in Table 6.6. Active site/substrate "mapping" was introduced earlier (Figure 6.15), where the scissile glycosidic bond is in register at subsites $-1/+1$. With few exceptions, one or two hydrophobic residues of the enzyme interact with the C_5-hydroxyl-methylenic group of the -1 substrate residue to provide a transition-state stabilizing "hydrophobic platform" [79].

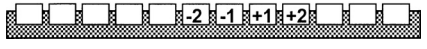
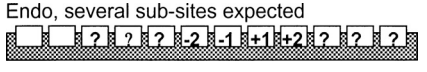
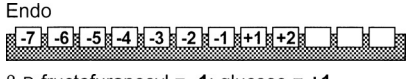
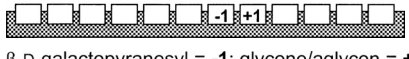
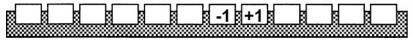
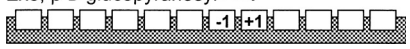
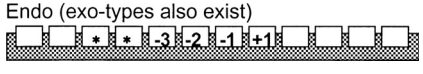
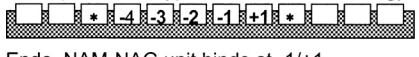
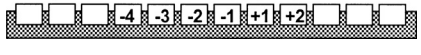
6.3.2.1 Starch-Transforming Enzymes

Enzymes acting on starch are primarily used for commodity applications, such as the production of corn syrups, dextrins, high fructose corn syrup, and other sweeteners such as maltose and glucose syrups. Starch transformations are also desirable to a more limited extent in baked goods, and exogenous glycosidases are added for purposes of retarding staling and facilitating yeast leavening.

6.3.2.1.1 α-Amylase [116,131,141,147]

The amylases are used to hydrolyze starch (mostly from corn) into smaller dextrins and thereby "thin" starch suspensions. α-Amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) is an endo-acting, $\alpha \rightarrow \alpha$-retaining enzyme principally responsible for rapidly reducing the average molecular weight of starch polymers. It is the representative member of family 13 glycosidases, several of which are used in starch processing. This family is characterized by having at least three separate domains within the protein, one for catalysis, another to serve as a granular starch binding site, and the third to provide for calcium binding and to link the other two domains. The molecular size of the enzyme from various sources (over 70 sequences have been reported) typically ranges 50–70 kDa (although some can approach 200 kDa). α-Amylases bind Ca^{2+} at multiple sites, the most important being near the active site cleft in a manner that stabilizes secondary and tertiary structure. Ca^{2+} is tightly bound and serves to broaden the pH stability of the enzyme to between pH 6 and 10, and the thermal stability of the α-amylase is quite dependent on source. The active site is comprised of at least five subsites (positions -3 to $+2$, Table 6.6; cf., Figure 6.15c), and requires a substrate of at least three glucose units in length. Of three conserved residues at the active site (porcine pancreatic α-amylase as reference), ASP_{197} is the nucleophile that forms the covalent glycosyl–enzyme intermediate, GLU_{233} is situated at the $+1$ subsite and participates as the general acid catalyst, and ASP_{300} serves to coordinate with C2—OH and C3—OH of the substrate unit at the -1 subsite to affect substrate strain/stress. Conserved HIS_{299} and HIS_{101} are involved in substrate binding and transition-state stabilization to collectively reduce E_a by 5.5 kcal mol^{-1}. HIS_{201} interacts with the catalytic GLU_{233} residue to shift pH optimum from 5.2 to 6.9. Because of the critical contribution of HIS residues to activity and pH-activity profile, HIS was long thought to be involved in the mechanism of α-amylase action. The pH optimum is also dependent on length of the substrate,

TABLE 6.6
Catalytic Properties of Glycosyl Hydrolases

Enzyme	Bond Selectivity	Product Selectivity[a]	Catalytic Residues[b]	Substrate Subsite Mapping[c]
α-Amylase	α-1→4 Glucose	RET $\alpha\rightarrow\alpha$	GLU_{233}, ASP_{300} (acid, nucl/base)	Endo 
β-Amylase	α-1→4 Glucose	INV $\alpha\rightarrow\beta$	GLU_{186}, GLU_{380} (acid, base)	Exo 
Pullulanase	α-1→6 Glucose	Likely RET $\alpha\rightarrow\alpha$	Not known—possibly GLU_{706}, ASP_{677} (acid, nucl/base)	Endo, several sub-sites expected 
Glucoamylase	α-1→4 (α-1→6) Glucose	INV $\alpha\rightarrow\beta$	GLU_{179}, GLU_{400} (acid, base)	Exo 
Cyclomaltodextrin transferase	α-1→4 Glucose	RET $\alpha\rightarrow\alpha$	GLU_{257}, ASP_{229} (acid, nucl/base)	Endo 
Invertase	β-1→2 Fructose	RET $\beta\rightarrow\beta$	GLU_{204}, ASP_{23} (acid, nucl/base)	β-D-fructofuranosyl = **-1**; glucose = **+1** 
β-Galactosidase	β-1→4 Galactose	RET $\beta\rightarrow\beta$	[d]GLU/Mg^{2+}, GLU_{537} (acid, nucl/base)	β-D-galactopyranosyl = **-1**; glycone/aglycon = **+1** 
β-Glucosidase	β-1→4, β-1→Aglycon glucose	RET $\beta\rightarrow\beta$	GLU_{170}, GLU_{358} (acid, nucl/base)	Exo, β-D-glucopyranosyl = **-1** 
Polygalacturonase	α-1→4 Galacturonate	INV $\alpha\rightarrow\beta$	$ASP_{180,201,202}$ likely acid/base residues	Endo (exo-types also exist) 
Xylanase	α-1→4 Xylose	RET $\beta\rightarrow\beta$	GLU_{172}, GLU_{78} (acid, nucl/base)	Endo (some exo-types exist, some inverting) 
Lysozyme	α-1→4-NAM–NAG[e]	RET $\alpha\rightarrow\alpha$	GLU_{35}, ASP_{52} (acid, nucl/base)	Endo, NAM-NAG unit binds at -1/+1 

[a] RET, retaining; INV, inverting.
[b] Reference enzyme cited in text.
[c] ?, subsite uncertain; *, some enzymes exhibit this subsite.
[d] Catalytic group probable but remains uncertain.
[e] *N*-acetylmuramate–*N*-acetylglucosamine repeating unit.

and action on maltooligosaccharides that do not fully occupy the five binding subsites has a narrower optimum pH range. Other conserved nonpolar residues are TRP, TYR, and LEU, which are involved in substrate and starch granule binding through hydrophobic stacking interactions [33,141].

There are several sources of α-amylases, most of which are microbial, although malt (barley or wheat) amylases are available. The typical end products of α-amylase action are branched α-limit dextrins and maltooligosaccharides of 2–12 glucose units, predominantly in the upper end of this range [141,142]. Starch is rapidly reduced in viscosity because of the random nature of hydrolysis, quickly reducing the average molecular mass of amylose/amylopectin chains. Among microbial amylases, optimum parameters are generally found within the ranges of pH 4–7 and 30–130°C [85]. A few amylases with alkaline pH optima of 9–12 evoke particular interest, not insofar as food processing aids, but from the prospect that an alternative may exist to the widely conserved glycosidase feature of the ASP/GLU dyad. Common commercial sources for starch transformation include the α-amylases from *Bacillus* and *Aspergillus* species. The *Bacillus* α-amylases are thermostable and can be used at 80–110°C at pH 5–7 and 5–60 ppm Ca^{2+} [142]. Fungal (*Aspergillus*) enzymes function optimally at 50–70°C, pH 4–5 and ~50 ppm Ca^{2+} [85,142]. While the fungal α-amylases are also endo-acting, they tend to favor the accumulation of shorter maltooligosaccharides ($n = 2$–5) as the end products of starch liquefaction [127]. A unique "maltogenic" *Bacillus* α-amylase (EC 3.2.1.133) has also been identified [26], and while maltose production is more commonly associated with the action of β-amylases (see next section), the maltogenic α-amylases appear to yield elevated maltose levels through either prolonged (exhaustive) hydrolysis of starch or by multiple ("processive") hydrolytic episodes on a bound amylose chain before it completely dissociates from the active site [33].

6.3.2.1.2 β-Amylase [85,116,127,141]

β-Amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2) is an $\alpha \rightarrow \beta$-inverting, exo-acting glycosidase that liberates maltose units from the nonreducing ends of amylose chains, and is a member of glycosidase family 14. Extensive action of β-amylase on starch yields a mixture of maltose and β-limit dextrins, the latter retains the α-1,6-branch points and remaining linear portions that are inaccessible (by steric constraints) to the enzyme. β-Limit dextrins are of greater average molecular mass than α-limit dextrins because the exo-acting β-amylase cannot act past the α-1,6 branch points, whereas α-amylase can, being an endo-acting enzyme. β-Amylases from soybean, sweet potato, and *Bacillus* spp. are among the best characterized; plant enzymes are ~56 kDa (sweet potato enzyme is a tetramer) while microbial enzymes range from 30 to160 kDa. β-Amylase is unique in that it has a single domain structure, instead of the multidomain structure of other amylolytic glycosidases. The catalytic residues (soybean β-amylase as reference) are GLU_{186} (general acid) and GLU_{380} (general base), which are separated by 10–11 Å and buried in a deep pocket. The binding of substrate causes a lid to close providing for an estimated 22 kcal mol^{-1} of favorable binding energy and shielding the active site from solvent. This likely intensifies dipole forces that facilitate catalysis and provides another example of "induced fit" mechanism. There are four substrate binding subsites with the catalytic GLU residues oriented on opposite faces of subsite -1. HIS_{93} is positioned at subsites -1 and -2 and may confer pH sensitivity on the alkaline side. The equivalent of two maltose units bind at the active site (subsites -2 to $+2$) and this property may confer how close the enzyme can act toward the branch points in starch. At one time, CYS residues were believed to be involved in catalysis, but point mutations have since revealed them to have little catalytic function, although they may have a role in enzyme conformational stability. While the plant enzymes cannot bind and digest raw starch, some of the microbial enzymes have separate protein domains that confer this ability. β-Amylase is subject to competitive inhibition by α-cyclodextrin, and this appears to be mediated by LEU_{383} forming an inclusion complex and blocking access to the active site. β-Amylases generally have more alkaline pH optima (pH 5.0–7.0) than α-amylases, do not require Ca^{2+}, and exhibit temperature optima in the range of 45–70°C, depending on source (microbial sources being more thermostable).

6.3.2.1.3 Pullulanase [131,141,147]

Type I pullulanases (EC 3.2.1.41, pullulan 6-glucanohydrolase) are referred to as "debranching" enzymes or "limit dextrinases," since they hydrolyze dextrins containing the α-1,6-glucosidic bonds constituting the branch points of amylopectin. Pullulanase is present in many bacteria, some yeast, and cereals, and while sequence analysis places it in the α-amylase family 13 ($\alpha \rightarrow \alpha$-retaining enzymes), structural features have not been reconciled. At this point, the active site residues (*Klebsiella pneumoniae* enzyme as reference) appear to be GLU_{706} (acid), ASP_{677} (nucleophile/base) with ASP_{734} assisting (Table 6.6). Pullulanase is characterized (and named trivially) by its ability to act on pullulan, a repeating unit of [α-D-Glc-(1$\rightarrow$4)-α-D-Glc-(1$\rightarrow$6)-α-D-Glc-(1$\rightarrow$4)-α-D-Glc]. Pullulanase can act on larger, but not smaller fragments than pullulan, acts slowly on amylopectin, and prefers the limit dextrins that are produced during advanced stages of starch liquefaction and saccharification [147]. Products of pullulanase action are linear glucooligosaccharides as small as maltose. Pullulanases are commonly obtained from *Klebsiella* and *Bacillus* spp., have masses of $\sim$100 kDa, upper temperature limits of 55–65°C, and optimal pH 3.5–6.5 with no known requirement for cofactors (although some are activated by Ca^{2+}). Pullulanases from plant sources are also referred to as limit dextrinases, and germinated or malted grains are the richest sources, especially barley. Type II pullulanases (or amylopullulanases, EC 3.2.1.41 or 3.2.1.1) are principally microbial in origin, have combined α-amylase–pullulanase activity, and can hydrolyze both α-1,4 and α-1,6 linkages in starch. Other related enzymes are neopullulanase (EC 3.2.1.125) and isopullulanase (EC 3.2.1.57), which act on the α-1,4 linkages in pullulan toward the nonreducing and reducing ends of the branch point, respectively, to yield α-1,6-branched trisaccharides panose and isopanose.

6.3.2.1.4 Glucoamylase [85,116,141]

Glucoamylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.3), also known trivially as amyloglucosidase, is an $\alpha \rightarrow \beta$-inverting, exo-acting enzyme (solely comprising glycosidase family 15) that hydrolyzes glucose units from nonreducing termini of linear starch fragments. Although glucoamylase is selective for the α-1,4-glucosidic linkage, it can act slowly on the α-1,6 bond characteristic of amylopectin and pullulan. Thus, the exclusive product of exhaustive glucoamylase digestion is glucose. It has structural and mechanistic features similar to α-amylase, including respective acid and base catalytic GLU_{179} and GLU_{400} residues (*Aspergillus* spp. enzyme as reference), a separate starch binding domain and short linker domain. Some glucoamylases can act on native (raw) granular starch. Two $TRP_{52,120}$ residues assist catalysis by H-bonding to GLU_{179}, enhancing its acidity. The catalytic domain has five subsites other than the scissile glycone residue at -1 (see Figure 6.15a), and subsites $+1$ to $+5$ all exhibit $-\Delta G$ for binding (favorable), especially at subsite $+1$. Since the ΔG is accretive for the subsites, the enzyme has greater reaction selectivity for the longer of the C2$-$C6+ linear glucooligosaccharides. This pattern of selectivity is conducive to obtaining processive and exhaustive hydrolysis of short amylose segments to glucose. The oligomeric substrate must enter a "well" to get access to the active site, and because of these steric constraints, dissociation and rebinding of remaining substrate is the rate-limiting step (instead of the hydrolysis step).

Glucoamylases are sourced primarily from bacteria and fungi [85]. They range in mass from 37 to 112 kDa, can exist as multiple isoforms, have no cofactors, and exhibit optima in the range of pH 3.5–6.0 and 40–70°C. The *Aspergillus* glucoamylase is commonly used and it is most active and stable at pH 3.5–4.5, with an optimum temperature range of 55–60°C [141]. The *Rhizopus* enzyme is of interest because one isoform can also readily hydrolyze α-1,6-branch points [85]. Glucoamylases are relatively slow acting glycosidases relative to others involved in starch transformation, and processing schedules have evolved to accommodate this feature.

6.3.2.1.5 Cyclomaltodextrin Glucanotransferase (Trivially, Cyclomaltodextrin Transferase) [116,141,142]

Cyclomaltodextrin glucanotransferase (CGT, 1,4-α-D-glucan 4-α-D-[1,4-α-D-glucano]-transferase [cyclizing], EC 2.4.1.9) catalyzes hydrolysis as well as intra- and intermolecular transglycosylation

reactions. The cyclization reactions yield the hexa- (α), hepta- (β), and octa- (γ) saccharides more commonly known as cyclodextrins. CGT is an $\alpha \rightarrow \alpha$-retaining, endo-acting enzyme belonging to family 13 of the glycosidases, and has two additional protein domains beyond the three observed for α-amylase, including additional substrate (specifically maltose) binding sites. The multiple binding sites allow interaction with raw starch (although CGT is not very active on raw starch), and helps guide linear starch fragments into the active site groove. CGTs are from microbial sources and typically monomeric with ~75 kDa mass. The catalytic residues (*Bacillus circulans* enzyme as reference) include ASP_{229} (base/nucleophile) and GLU_{257} (general acid), while ASP_{328}, $HIS_{140,327}$ have roles in substrate binding and transition-state stabilization, ARG_{227} orients the nucleophile, and HIS_{233} coordinates with required Ca^{2+} (as with some α-amylases). There are nine subsites at the active site, -7 to $+2$, consistent with β-cyclodextrin being the favored product of intramolecular cyclization (Table 6.6).

Although cyclodextrins are the primary commercial products prepared by CGT, CGT is quite promiscuous in its substrate and product selectivity, as it can catalyze a diversity of reactions, including hydrolysis, cyclization, disproportionation, or coupling. For example, it can react with glucose and starch to form maltooligosaccharides of various chain lengths, as well as couple sugars (many monosaccharides are recognized) with alcohol groups such as those of ascorbic acid and flavonoids. These latter processes offer potential for preparing novel compounds of unique functionalities in food systems. CGTs typically exhibit optima at pH 5–6, and temperature optima have been improved from 50–60°C to 80–90°C in recent years by the introduction of thermostable forms. Different sources of CGT favor different cyclodextrins (hexa-, hepta-, or octa-oligomers) as the principal product.

6.3.2.1.6 Applications of Starch Transformation

Starch hydrolysis: Industrial starch transformation begins with a starch slurry substrate of 30–40% solids at nascent pH of 4.5 (Figure 6.19). "Liquefaction" following pH adjustment to 6.0–6.5 of the starch occurs by briefly heating to 105°C (to gelatinize the starch) and then tempering to 90–95°C for 1–3 h in the presence of a thermostable (bacterial) α-amylase and added Ca^{2+}. This yields a mixture of linear and branched dextrins (maltodextrins) with extent of hydrolysis ranging from 8 to 15 DE (DE = dextrose equivalence), and this is sufficient to prevent gelation of starch on cooling for subsequent steps (hence the term liquefaction). From this point there are three alternative steps. One is for the production of 15–40 DE maltodextrins (corn syrups, used as thickening, bulking, and viscosity building), which is conducted by further exposure to amylase (in some cases acid [HCl] is used to initially liquefy the gelatinized starch). Two other streams lead to sweetener production, which also requires a reduction in temperature to ~60°C and pH to 4.5–5.5 to accommodate optimal conditions of the enzymes used. For conversion to a 95–98% glucose syrup (95 DE), solids are reduced to 27–30% and treated with glucoamylase (which is often used as an immobilized enzyme column), with or without pullulanase, for 12–96 h. More than 95% glucose syrup can then be refined, concentrated to 45% solids, and treated with an immobilized xylose (glucose) isomerase column at pH 7.5–8.0 and 55–65°C with added Mg^{2+} to generate a high-fructose corn syrup of 42% fructose (52% glucose), which can be further refined and/or enriched to a 55% fructose syrup. The other sweetener produced from the liquefied starch is facilitated by added fungal (maltogenic) α- or β-amylase, with or without added pullulanase, to yield a range of maltose (30–88%) syrups for use in confections. Depending on the source of maltogenic amylase selected, the predominant maltooligosaccharides that accumulate in the product mixture can range between two and five glucose units.

Two other types of nonsweetener products prepared from the original starch slurry involve the action of various α-amylases added before the starch is progressively heated to the point of gelatinization. This leads to a controlled (DE 3–8) pattern and degree of hydrolysis that yield large dextrins (generically called starch hydrolysis products) that can form thermoreversible gels and behave as fat mimetics. Much of the details of preparing these products are in the patent literature, but the process

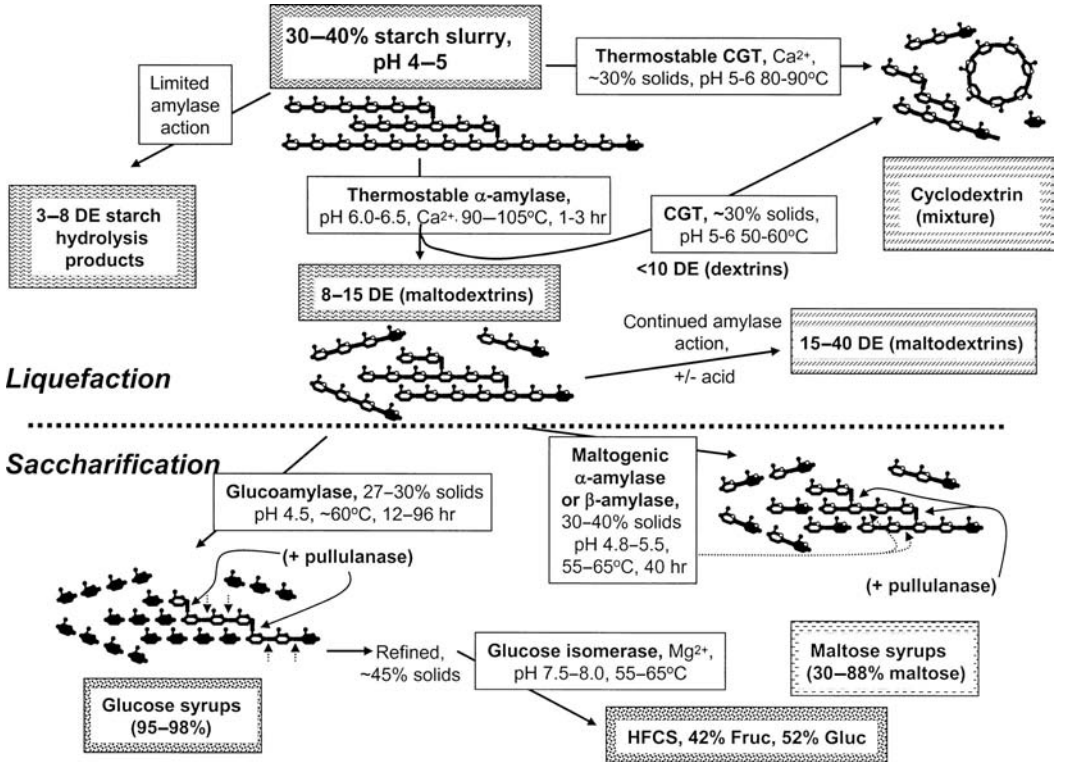


FIGURE 6.19 Commercial starch transformation by enzyme processing. Filled glucose units are reducing ends, and provide the basis by which DE is measured.

generally involves limited amylase action over a range of temperatures [127]. Alternatively, thermostable CGT can be added to the native starch slurry after adjusting pH to 5–6 and then incubating at 80–90°C. Total yields of cyclodextrin from CGT action on starch are inversely proportional to the concentration of starch and degree of liquefaction [147]. Thus, cyclodextrin production in commercial processes is often conducted at starch levels of ~30% solids (1–33% have been reported in patent literature [124]) as a compromise between percentage yield (efficiency) and total yield (production). Thermostable CGT can both hydrolyze native (gelatinized) starch in the presence of added Ca^{2+} and transglycosylate (cyclize) resulting fragments. Nonthermostable CGT can also be used but requires prior and limited digestion of starch to afford liquefaction (to about 10 DE to prevent gelling) after which CGT is added at reduced temperatures (50–60°C). Cyclodextrin yields can be enhanced by pre- or cotreatment of starch with a debranching enzyme and by incorporating complexing agents (solvents or detergents) to direct the reaction toward one or more of the cyclodextrin species [124,147].

Going forward, efforts to improve starch processing and transformation will focus on extending pH stability (to pH 4–5) and reducing Ca^{2+} requirement of α-amylase, and enhancing the ability to digest raw starch by β-amylases [85,131]. For all enzymes involved, enhancing thermal stability is of importance as this will create efficiency in processing as well as promote single-step processing. In addition, discovering the determinants of product selectivity of reactions to obtain preferred products or product distributions will remain a priority.

Baking and baked goods [26,95,131]: Virtually all of the glycosidases discussed earlier have been added for some benefit in baking applications, and the α-amylases have been used the most. Initially amylases were believed to function primarily by mobilizing fermentable carbohydrate for yeast. They are also added to doughs to degrade damaged starch and/or supplement endogenous amylase

activities of poor quality (in terms of baking) flours. However, it is now recognized that amylases added directly to the dough will reduce dough viscosity, and improve loaf volume, crumb softness (antistaling), and crust color. Most of these effects can be attributed to partial hydrolysis of starch during baking as the starch gelatinizes. Lowered viscosity (thinning) helps promote volume and texture by allowing reactions involved in dough conditioning and baking to occur faster (mass transfer effect). The antistaling effect is believed to be conferred by limited hydrolysis of amylose and especially amylopectin chains in a manner that retards the rate at which they can retrograde, and this remains the primary reason why α-amylases are added to baked goods today.* Overdosing of α-amylases leads to gummy or sticky textured breads, and this is associated with the accumulation of branched maltodextrins of 20–100 DP. Thus, care must be exercised to apportion the right amount of amylase for a specific product, and amylases should not survive the baking process or unwanted residual activity will occur postproduction. This has been done by matching the temperature stability and amount of added amylase to the particular application to control the extent to which the amylase acts and persists during the baking cycle [53]. More recently, the maltogenic types of α-amylases have been recognized as being superior as antistaling agents, since they tend to form shorter maltooliogosaccharides (DP 7–9) and large dextrins (which are plasticizers) than those arising from the endo-action by conventional α-amylases. Thus, maltogenic amylases tend to keep the gelatinized starch network in bread intact (soft, but not gummy), and the slight reduction in size of starch chains maintains elasticity of the crumb while being sufficient to retard staling.

Brewery and fermentations [141,142]: Starch hydrolases have long been recognized as essential enzymes in the brewing industry, originating with the 1833 finding of "diastatic" activity in malted (germinated) grains, leading to the commercialization of α- and β-amylases. However, amylases endogenous to malted grain are insufficient to mobilize all of the fermentable carbohydrate because they are of insufficient concentration, lack thermal stability for the processes involved, and/or there are endogenous inhibitors present in the grains. Thus, α- and β-amylases, glucoamylase and pullulanase, and cell wall hydrolyzing enzymes are added (almost exclusively from microbial sources) to maximize the availability of fermentable carbohydrate. Glucanases and xylanases (discussed later) are added to hydrolyze glucans (similar to cellulose, but with β-1,3 and β-1,4 linkages) and xylans (predominantly xylose polymers, the major hemicellulose component in cell walls). The added α- and β-amylases are used to complete the degradation of starch to α- and β-limit dextrins that the thermally labile malt amylases cannot achieve. The remaining limit dextrins provide body to the final product. However, limit dextrins can be rendered fermentable by added glucoamylase (and/or pullulase), and beers produced with this enzyme are lower in calorie ("light"). Exogenous enzymes are added during (or right after) the "mashing" step, which is conducted at moderate temperatures (45–65°C), and they are destroyed during the subsequent "wort" boiling stage.

6.3.2.2 Sugar Transformation and Applications

6.3.2.2.1 Glucose Isomerization

Xylose (glucose) isomerase (EC 5.3.1.5, D-xylose ketol-isomerase) is one of the most widely recognized enzymes in sweetener production from corn starch, and it has only been found in microorganisms [3,127,141]. Although it is most selective for xylose, it reacts efficiently enough with glucose in an equilibrium isomerization reaction yielding fructose that it has become one of the most important industrial enzymes, used for the production of high-fructose corn syrup (sweetener). The mechanism of this enzyme and important active site residues were discussed in detail in Section 6.2.4.2. The enzyme exists as homotetramers, ranging 170–200 kDa, with two essential metal cofactors (one each catalytic and structural) per subunit (Mn^{2+}, Mg^{2+}, and Co^{2+} are common). The enzyme is

* Estimates of value of disposed baked goods because of staling in the United States in 1990 was about US$1 billion [53].

commercially available (principally from *Streptomyces* spp.) as an immobilized form and packed in a column through which glucose syrup is infused. Typical operating conditions make use of ion-exchange and charcoal to refine 40–50% solids glucose syrup (93% solids as glucose) resulting from starch saccharification (Figure 6.19). The pH is adjusted to ~7.5 (representing a compromise between maximal stability at pH 5–7 and maximal activity between 7 and 9), 1.5 mM Mg^{2+} is added, and the syrup is perfused through the reactor for an appropriate residence time to obtain the desired conversion at 55–65°C (even though temperature optima is 75–85°C). The temperature is a compromise between maximizing enzyme stability (to allow functioning for several weeks to months), reducing viscosity, preventing microbial growth, and limiting Maillard-type reactions (glycation) of enzyme amino side chains, resulting in inactivation. The greatest limitation in the industrial use of glucose isomerase is thermal instability. Depending on the conditions of use, a glucose syrup (DE ~95) can be converted into a 42–45% fructose syrup (balance glucose). Operating the enzyme at more elevated temperatures would favor the yield of fructose (based on the temperature dependence of the equilibrium constant), and molecular biology efforts are being used to engineer greater thermal stability.

6.3.2.2.2 Glucose Oxidation [117,141]

Glucose oxidase (EC 1.1.3.4, β-D-glucose:oxygen 1-oxidoreductase) is obtained primarily from *Aspergillus niger*. It is a dimeric glycoprotein of 140–160 kDa, with a deep binding pocket that hosts glucose through 12 H-bonds and multiple hydrophobic interactions, accounting for its sugar specificity. Despite this, the K_M for glucose is rather high at ~40 mM, but this is compensated for by the high turnover/catalytic rate of the reaction. The enzyme is quite stable up to 60°C and over a pH range of 4.5–7.5, allowing a diversity of conditions to employ glucose oxidase as a processing aid. Glucose oxidase is principally used to deplete egg whites of glucose and reduce the potential for Maillard browning on dehydration and storage. Egg whites must first be adjusted in pH from ~9 to <7 with citric acid before glucose oxidase is added along with H_2O_2 (to serve as a reservoir for O_2 provided by often coexisting catalase activity), at 7–10°C for up to 16 h, prior to spray drying [77]. Other potential uses of using glucose oxidase for removing oxygen in liquids or within packages, or generating gluconic acid (an acidic fermentation product and chemical leavening agent) have not been widely adapted. Glucose oxidase can also be used to generate H_2O_2 as an antibacterial agent (in toothpaste), or as a dough conditioner (strengthener) by providing oxidants where it may serve as a "natural" agent to replace bromates to induce disulfide linkages in gluten [142].

6.3.2.2.3 Sucose Hydrolysis (Inversion) [116,141]

Invertase (EC 3.2.1.26, β-D-fructofuranoside fructohydrolase) has long been the subject of study, and yeast invertase was the enzyme selected by Michaelis–Menten (1913) to generate the data to construct their kinetic model. About 40 invertases have been sequenced, and they exist as isoforms in plant tissues and microorganisms, being monomeric or oligomeric proteins with molecular masses ranging from 37 to 560 kDa. Many are glycoproteins, and plant isoforms are often referred to as acid- or neutral-, and alkaline-type invertases to reflect conditions of optimal activity (pH 4–5 and 7–8, respectively). Invertase is a $\beta \rightarrow \beta$-retaining glycosyl transferase, and the common name "invertase" reflects the ability of the enzyme to change ("invert") the optical rotation (polarimetry) of a sucrose solution and not the stereochemistry of its action (Table 6.6). The enzyme is unique in that it can withstand and remain active at high osmolalities (up to 30 M sucrose). The catalytic residues (yeast enzyme) are GLU_{204} (acid) and ASP_{23} (nucleophile/base). Substrate selectivity is toward β-D-fructofuranosyl glycosides, the most important one being sucrose. Invertase (from yeast) is used as an exogenous enzyme primarily in the production of soft-centered confections and to produce artificial honey from sucrose. For confectionary use, the enzyme can either be injected into coated confections or mixed with the granular sugar mixture (fondant) immediately before being coated. Allowing the confection to stand provides time for invertase to act on sucrose and cause viscous liquefaction of the center.

6.3.2.2.4 Lactose Hydrolysis [116,141]

β-D-Galactosidase (EC 3.2.1.23, β-D-galactoside galactohydrolyase or lactase) is found in mammals (intestinal tract) and microorganisms, and belongs to family 2 of the glycosyl hydrolases. These enzymes typically exist as tetramers of polypeptide chains ranging in mass from ~90 to 120 kDa, and the enzyme (*lacZ*) from *Escherichia coli* is representative of lactases. Each dimeric unit contributes the Mg^{2+} binding site and two catalytic residues (one from each polypeptide); thus, there are two active sites for each tetramer, and the binding pocket is a deep cleft at the interface of the polypeptide chains. The catalytic nature of the GLU_{537} residue (nucleophile/base, *E. coli*) is now well established (Table 6.6), although there remains some ambiguity regarding the general acid catalytic unit(s). The acid group appears to be within a cluster of other residues implicated as being essential to catalysis, including GLU_{461}, MET_{502}, and TYR_{503}, as well as two $GLU_{416,461}$ and HIS_{418} that coordinate with Mg^{2+}. Conventional thinking is that either the Mg^{2+} or GLU_{461} constitutes the putative general acid catalytic function, although they may act in concert in this regard. Many β-D-galactosides are acted upon by lactase, indicating rather strict specificity for the glycone residue (-1 subsite), although a comprehensive analysis of subsite relationships appears to be lacking. H-bonding of enzyme HIS_{540} with C2—OH, C4—OH, and C6—OH confers transition-state stabilization and may have a role in glycone specificity. The broad specificity toward the nongalactosyl residue has led to the use of a model, chromogenic substrate, *o*-nitrophenyl β-D-galactoside, for routine and facile assay of the enzyme. Consistent with being a $\beta \rightarrow \beta$-retaining enzyme, β-D-galactosidase can also catalyze transglycosylation reactions of galactose with other sugars (lactose, galactose, glucose) through β-1,6 linkages, to form unusual oligosaccharides of 2–5 DP.

The enzyme from microbial sources offers a wide range of pH optima (5.5–6.5 for bacteria, 6.2–7.5 for yeasts, and 2.5–5.0 for fungi) for commercial applications. Temperature optima are 35–40°C for the bacterial and yeast enzymes and up to 55–60°C for the fungal enzymes. The fungal enzyme is the only form not activated by Mg^{2+} or Mn^{2+}. This operational diversity allows for use of microbial β-D-galactosidases in acidic foods (acid whey, fermented dairy foods) as well as in milk and sweet whey. The enzyme is subject to inhibition by product (galactose), Ca^{2+} and Na^{+}. Lactose hydrolysis can be used to enhance sweetening power, fermentable substrates, and reducing sugars; reduce the incidence of lactose crystallization (e.g., "sandiness" in ice cream); and allow consumption of dairy products by lactose-intolerant individuals (such individuals lack sufficient lactase, which in mammals exists as a lactase-phlorizin hydrolase, an enzyme with two active sites and functions). Lactose hydrolysis can take place in fluid milk, and this is accomplished by direct addition (batch processing) of the yeast enzyme, which can achieve ~70% hydrolysis; the enzyme is subsequently destroyed by pasteurization [142]. Whey or whey permeate solids can be processed by immobilized enzyme reactors using the *Aspergillus* β-D-galactosidase, achieving ~90% lactose hydrolysis.

6.3.2.2.5 Other Glycosidases

β-Glucosidases (EC 3.2.1.21; β-D-glucoside glucohydrolase) are a diverse group of enzymes comprising portions of families 1 and 3 of the glycosyl hydrolases. β-Glucosidase is a $\beta \rightarrow \beta$-retaining enzyme, with the respective acid and nucleophilic residues being GLU_{170} and GLU_{358} (at 5.5 Å spacing, reference enzyme from *Alcaligenes faecalis*) (Table 6.6). β-Glucosidases come from many microbial and plant sources, with the most widely available enzyme being that from almond (also called "emulsin"). β-Glucosidases tend to have broad pH (4–10) stability, and are optimally active at pH 5–7, depending on source. Upper practical temperature ranges are 40–50°C, and while the enzyme is sensitive to sulfhydryl reagents (implying a stabilizing role of CYS), its compact structure renders the enzyme quite resistant to proteolytic attack. β-Glucosidases can hydrolyze sugars (such as cellobiase on cellobiose), thioglycosides, and β-D-glucosides of alkyl and aryl groups (which constitute the aglycon portion). Action on the latter types of β-glucosides can generate aromatic compounds in beverages made from fruits (wine and juices) as well as tea [141,143]. Removal of bitterness (naringin) from citrus juices is afforded by β-glucosidases, and such activity may be present in pectinase preparations used in fruit extract/juice preparation. Some endogenous β-glucosidases

may be responsible for the emanation of bioactive agents, such as HCN (from cyanogenic glucosides linamarin in cassava and lima beans; dhurrin in sorghum; amygdalin in almonds, peaches, and apricot pits), and the anticarcinogenic and goiterogenic (and pungent/bitter flavored) isothiocyanates from glucosinolate substrates in Brassicas (by the enzyme myrosinase, discussed later). While some glycosidases in "pectinase" preparations may liberate aromatic flavors from precursors in treated fruit juices, detrimental effects include the loss of anthocyanin-based coloration, and the production of adverse ("rotten-fruit") flavors from release of ferulic acid [143].

Isomaltulose synthase is an enzyme with both glycosyl hydrolase and transglycosylation activity. The active site residues include ASP_{241} and GLU_{295} (*Klebsiella* spp. LX3 enzyme as reference), as nucleophile/base and acid, respectively [150]. The two-step reaction pathway involves initial hydrolysis of sucrose (α-D-glucosyl-1,2-β-D-fructose), followed by glycosylation of fructose at the C6—OH site to yield isomaltose (α-D-glucosyl-1,6-β-D-fructose). The net effect is an isomerization and both reaction steps occur at a single active site. An industrial process makes use of immobilized baterial cells [16] to produce isomaltulose (also called isomaltose), a noncariogenic sweetener, potential prebiotic agent, and substrate for hydrogenation to yield the disaccharide sugar alcohol known as Isomalt®.

α-Galactosidase (EC 3.2.1.22) is used to convert raffinose in sugar beet into sucrose to increase process yield by 3% and facilitate sucrose recrystallization. Mycelial pellets of *Motierella vinacea* are the source of the commercial enzyme [16].

6.3.2.3 Enzymic Pectin Transformation [141]

Pectin-degrading enzymes are categorized into three general types, polygalacturonase, pectate and pectin lyases, and pectin methyl esterase. The specific reactions caused by these three pectinase activities are shown in Figure 6.20 [11,91]. These enzymes are typically found in plants and microorganisms (especially fungi) and exist as multiple isoforms. Collectively, this group of enzyme activities comprises "pectinase" preparations, often derived from *A. niger*, which are used in most commercial applications for fruit and vegetable tissue processing, juice extraction, and clarification.

6.3.2.3.1 Polygalacturonase [11,91,141]

Polygalacturonases (galacturonide 1,4-α-galacturonidase, EC 3.2.1.15 for *endo*-acting form, and EC 3.2.1.67 and 3.2.1.82 for *exo*-acting forms) are $\alpha \rightarrow \beta$-inverting enzymes that belong to family 28 of the glycosyl hydrolases (Table 6.6). The endoenzyme from *A. niger* has three conserved $ASP_{180,201,202}$ that function as the general acid–base catalytic units but they appear to be within 4.0–4.5 Å and not at 9.0–9.5 Å apart common to inverting glycosidases. Prevailing view is that $ASP_{180,201}$ activates water as the nucleophile, while ASP_{202} protonates the leaving group and is assisted by HIS_{223} (also conserved), while a conserved TYR_{291} also assists catalysis. Four to six substrate binding subsites exist ($-5/-3$ to $+1$, depending on isoform) consistent with the endo-acting property, and this feature confers the minimum substrate size. Isoforms with greatest strength of binding (affinity) at the -5 subsite do not react randomly, but instead react processively by rebinding and repeatedly hydrolyzing a single chain. LYS_{258} is important at the -1 subsite and may confer the requirement for a Gal*p*A substrate residue (non-esterified) binding at this site through ionic interaction with the carboxylate group.

Fungal enzymes are most active over a pH range of 3.5–6.0 (as are plant enzymes), at 40–55°C, and have molecular masses ranging 30–75 kDa. The result of polygalacturonase action is the depolymerization of pectin and the progressive solubilization of polyuronide fragments. The practical outcome of such activity is that intercellular barriers (middle lamella) are broken down, and viscosity of pectin solutions becomes diminished as enzyme action is sustained. While exopolygalacturonases are also of fungal origin and available, they are nonactive when a methylated galacturonic acid residue binds at the $+1$ subsite, and since they are not efficient at depolymerization and viscosity reduction, they are of limited utility.

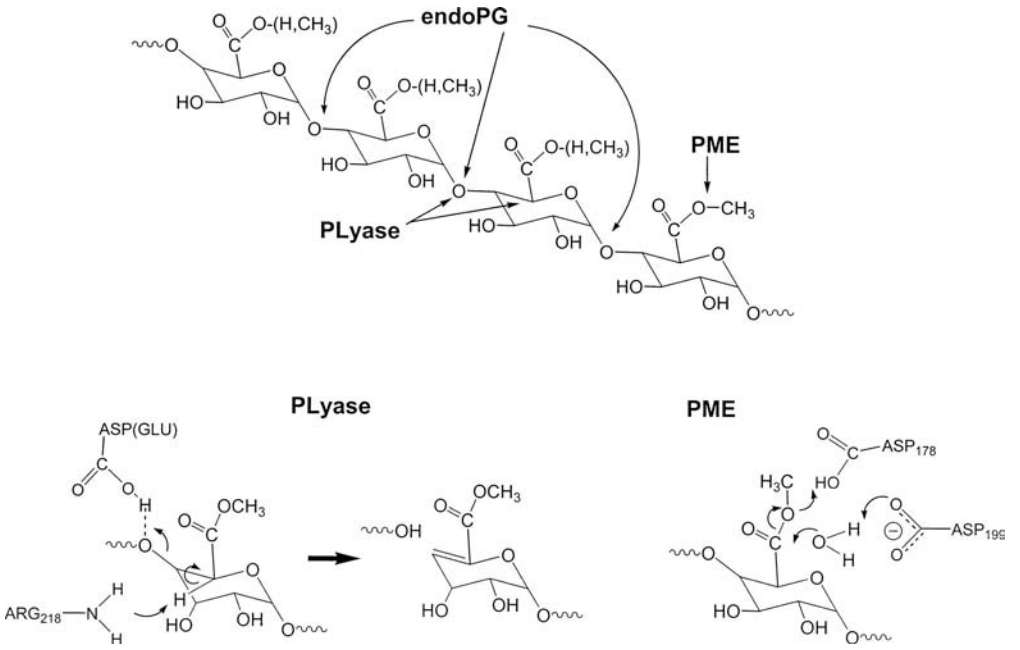


FIGURE 6.20 Site of action and reaction mechanism of pectin-degrading enzymes. (Adapted from Benen, J.A.E., et al. (1999). In *Recent Advances in Carbohydrate Engineering*, Gilbert, H.J., et al. (Eds.), The Royal Society of Chemistry, Cambridge, UK, pp. 99–106; Pickersgill, R.W. and Jenkins, J.A. (1999). In *Recent Advances in Carbohydrate Engineering*, Gilbert, H.J., et al. (Eds.), The Royal Society of Chemistry, Cambridge, UK, pp. 144–149; and Whitaker, J.R., et al. (Eds.) (2003). *Handbook of Food Enzymology*, Marcel Dekker, New York.)

6.3.2.3.2 Pectinesterase [91,141]

Pectin methyl esterases (EC 3.1.1.11, pectin pectylhydrolase) have been best characterized from fungi, although they are prevalent also in plant tissues. Collectively, these enzymes exist as multiple isoforms (acidic, neutral, alkaline) from a given source, range 25–54 kDa in mass, can have broad pH stability (within the general range of pH 2–10), and can have quite moderate thermal stability (40–70°C), depending on source. Fungal enzymes have optima between pH 4 and 6 while plant enzymes have more alkaline optima (pH 6–8) and often require submillimolar levels of Na^+. Being a carboxylesterase, the catalytic units and mechanism are expected to resemble the ASP–HIS–SER triad (as for lipases and serine proteases). However, two $ASP_{178,199}$ and one ARG_{267} residues (*Erwinia chrysanthemi* enzyme as reference) are conserved among pectin methyl esterases. One ASP is unprotonated and serves to activate nucleophilic water to attack the carbonyl carbon, while the other ASP is acidic and protonates an oxygen of the carbonyl (Figure 6.20). A noncovalent tetrahedral intermediate forms and collapses to yield the free acid and methanol. Studies on the *A. niger* enzyme suggest 4–6 glycone binding sites, and that demethylation cannot occur at the nonreducing terminus of a pectin fragment. Substrate/product selectivities vary among pectin methyl esterases (and isoforms) in terms of the preferred degrees of methylation of the pectin substrate, whether continued hydrolysis is favored on a single pectin chain, and whether hydrolysis is at random or at closely spaced sites.

6.3.2.3.3 Pectate Lyase [141]

Pectate lyase (EC 4.2.2.2, (1→4)-α-D-galacturonan lyase) and pectin lyase (EC 4.2.2.10, (1→4)-6-*O*-methyl-α-D-galacturonan lyase) also depolymerize pectin, with the former recognizing acidic residues adjacent to the scissile bond and the latter recognizing methyl-esterified residues adjacent to the scissile bond (the galacturonate residue to be attacked is positioned at the +1 subsite). Both

enzymes occur in multiple isoforms. Pectate lyase has a requirement for Ca^{2+} to coordinate the galacturonate group at the active site. These enzymes are prevalent in fungi and also found in bacteria (*E. chrysanthemi* enzymes are among the best known), while found to a limited extent in plants. For pectate lyases, pH optima are often in the alkaline range of 8.5–9.5, and low-methoxy pectins are preferred as substrate. Pectin lyases have pH optima ~6, in accord with the preference to act on acidic (protonated) or fully methoxylated forms of pectin, and *Aspergillus* spp. are common sources. Although pectin lyases do not require Ca^{2+}, this cation stimulates activity and shifts pH optimum to a more acidic region. Both lyases are stable up to ~50°C, and a conserved ARG_{218} (*E. chrysanthemi*) acts as the base in the reaction mechanism to abstract the proton from C5 (Figure 6.20), while the role of the acid group remains unknown. Exo-acting enzymes have small subsites (limited to −1 or −2 toward the nonreducing terminus) while endo-acting enzymes range in subsite topography from −2/+2 to −7/+3, depending on isoform. Because of the pH of vegetable and especially fruit tissues that are processed for juice, pectin lyases are generally more applicable as processing aids than are pectate lyases.

6.3.2.3.4 *Applications of Pectin-Degrading Enzymes [3,48,77,141,142]*

The common uses of pectinase preparations and related enzymes include tissue maceration, tissue liquefaction, enhanced recovery or extraction (juice or oil), clarification, and facilitated peeling (especially citrus fruits). Commercial "pectinase" preparations are often crude mixtures of several types of pectin-, cellulose-, and hemicellulose-degrading enzymes, and the evolution in "pectinase" applications will be toward more specific deployment of enzymes for specific purposes and products. In most cases, the enzyme treatments are conducted at 20–30°C for several hours for mild processes or at 40–50°C for 1–2 h for the more rigorous ones (maceration, liquefaction) at the pH of the juice or extract. Tissue processing begins with an initial crushing (milling) or coarse grinding of the tissue (Figure 6.21 [48,77,142]). The coarse tissue preparation is subjected to enzyme preparations that exclusively hydrolyze and deploymerize the middle lamella (pectic substances), as this will most

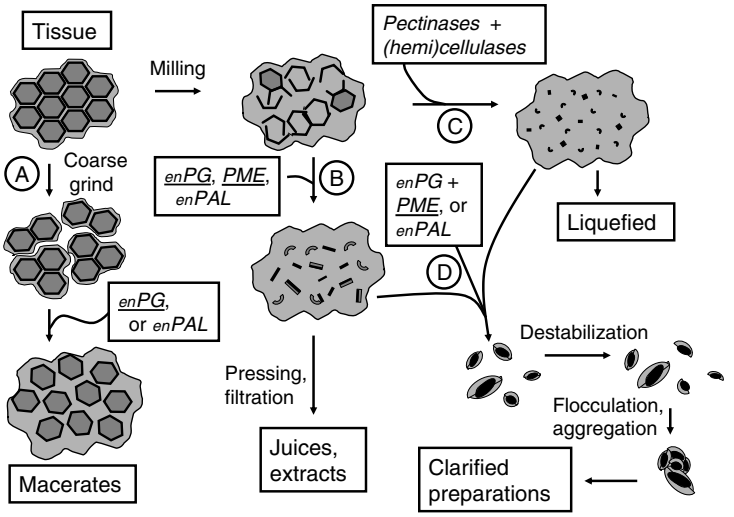


FIGURE 6.21 Commercial processing of fruit and vegetable extracts using pectinases. (Compiled from information contained in Godfrey, T. and West, S. (Eds.) (1996). *Industrial Enzymology*, 2nd edn., Stockton Press, New York; Nagodawithana, T. and Reed, G. (Eds.) (1993). *Enzymes in Food Processing*, 3rd edn., Academic Press, New York, p. 480; and Whitehurst, R.J. and Law, B.A. (Eds.) (2002). *Enzymes in Food Technology*, 2nd edn., CRC Press, Boca Raton, FL.)

readily liberate individual cells with intact cell walls (Figure 6.21a). Thus, endopolygalacturonase (especially) or endopectin lyases are most suitable and the resulting cell suspensions can be used in pulpy juices or nectars, infant foods, or ingredients for other products.

Other processing streams originate with a finer grinding or crushing of tissue (often by a hammer mill), where maximal expulsion of liquid, and sometimes pulpy matter, is the desired outcome. "Extracts" from pome fruits and berries often require addition of enzymes to convert the viscous to semigelled mash of crushed fruit (caused by partial solubilization of pectins and high water-holding capacity of solids) to maximize juice extraction during subsequent pressing (Figure 6.21b). Pectin-degrading enzymes capable of depolymerizing and degrading highly methoxylated pectins are most suitable, and include endopolygalacturonase and pectin methyl esterase in particular, while endopectin lyase may also be used. Juices prepared this way may be either clear or cloudy depending on the specific tissue and combination of enzymes used.

Liquefaction of fruit or vegetable material is used to convert the entire mass of tissue to a liquid product, and such a product does not require filtration or pressing (Figure 6.21c). This is accomplished with a robust combination of pectinases (polygalacturonase, pectin methyl esterase, and pectin lyases), cellulases (both exo- and endo-β-glucanases), and hemicellulases (acting on xylans, mannans, galactans, and arabinans). Once much of the middle lamella and cell wall material is "solubilized" (as much as 80%), the cells are easily burst by osmotic pressure or shear to release liquid contents. Liquefaction is used to convert many pulpy tropical fruits (mango, guava, banana), olives, and stored apples into juices or oleaginous extracts. These juices may be rendered cloudy or clear depending on the tissue and enzymes used.

The last major application of exogenous pectin and cell wall degrading enzymes is for juice or extract clarification (Figure 6.21d). This requires initiating events that destabilize any "cloud" in the juice or extract. Cloud may be desirable in some juices (e.g., orange), but not for those juices such as apple and grape, where transparent juices are preferred. Cloud is conferred by colloidal particles consisting of protein (positively charged at juice pH) coated with pectin (galacturonic acid residues are partially dissociated and negatively charged). Pectin-depolymerizing enzymes solubilize and disrupt the pectin layer, allowing protein to electrostatically interact with pectin layers of other particulates, leading to aggregation, flocculation, and providing for easy clarification. The optimum pH for this process has been estimated to be ~3.6, and clarification is most often facilitated by pectin methyl esterase, especially in combination with endopolygalacturonase, or pectin lyase alone for highly methoxylated pectins (apples). The role of pectin methyl esterase action is to yield sites for Ca^{2+}-induced cross-linking of particulates, leading to aggregates that easily settle. In some cases, clarified juices may undergo reversion haze; arabinose comprises ~90% of the polysaccharide material participating in this haze. Haze is minimized if endoarabinanase is included in the pressing and/or clarification treatment.

In citrus (orange) juices, a process called "pulp wash" calls for pectinases to reduce viscosity of the water extraction of residual pulp before it is added back to the initial juice expressed from the tissue. Citrus can also be enzyme peeled, by scoring the surface and vacuum-infusing pectinase for ~1 h at 20–40°C, as this allows the white spongy albedo to be digested leaving the "peeled" fruit easily segmented after removal of the skin (flavedo). Specific considerations of which process and enzymes to use for specific fruits, and the products to be prepared therefrom exist and are described in detail elsewhere [142].

6.3.2.4 Other Glycosidases

Xylanases (EC 3.2.1.8, β-1,4-D-xylan xylohydrolase) are largely $\beta \rightarrow \beta$-retaining glycosidases of families 10 and 11 (xylanases in other families exist), capable of hydrolyzing linear β-1,4-linked polymers of xylose (with various substitutive groups such as arabinose) [116,141] (Table 6.6). Multiple isoforms exist and these enzymes can be endo- or exo-acting (endo-acting are more important in foods). Xylans are a major hemicellulose component and together with cellulose they comprise the

bulk of the cell wall material in botanical products. Xylanases are found in plants (especially important in cereals), bacteria, and fungi, and they typically range in molecular mass from 16 to 40 kDa. The *B. circulans* enzyme has catalytic residues of GLU_{78} (nucleophile) and GLU_{172} (general acid/base) with the latter residue cycling between pK_a of 6.7 (free enzyme) and 4.2 (substrate-bound form). The xylanase A from *Pseudomonas fluorescens* has a substrate subsite topography of -4 to $+1$. In general, subsites range from 4 to 7 residues. Bacterial enzymes are sourced from *Bacillus*, *Erwinia*, and *Streptomyces* spp., while fungal enzymes are sourced from *Aspergillus* and *Trichoderma* spp. Bacterial enzymes have optima of pH 6.0–6.5, while fungal enzymes are most active at pH 3.5–6.0, depending on source, and most xylanases have broad pH stability within the range of pH 3–10. Optimum temperatures for activity range 40–60°C.

Xylanase enzymes are beneficial by depolymerizing water-unextractable arabinoxylan into water-soluble pentosans, the latter which have high water-holding capacity [95]. This increases the viscosity of the dough and leads to increased elasticity, gluten strength and final loaf volume. Excessive dosing of xylanases, or addition of xylanases that preferentially act on water-soluble arabinoxylans either have no effect or result in sticky dough (caused by excessive degradation of water-holding pentosans) with compromised performance. The combination of amylases and xylanases are particularly important in the formulation of frozen doughs [42].

Endoxylanases are one of the hemicellulases that are used in fruit and vegetable processing. Xylanases are also used in brewing to reduce viscosity of the wort in brewing, allowing ease in separation/filtration steps, reduced haze formation, and slightly improved process yields. Hemicellulases/xylanases from *Trichoderma* and *Penicillium* spp. have found use in a wet milling process to separate starch from gluten in grains, especially wheat [48].

Other cell wall degrading enzymes of importance are those that hydrolyze the β-1,4 and -1,3 linkages of glucans, collectively referred to as cellulases and glucanases [141]. These enzymes are added to assist fruit and vegetable tissue liquefaction processes, and to brewer's grain to enhance level of fermentable sugars, aid in filtering of spent grains from the wort, and reduce the incidence of "glucan haze" formation [3,142].

Lysozyme has been featured already in terms of mechanism of action (Equation 6.8, Figure 6.15b). It is an $\alpha \rightarrow \alpha$-retaining glycosidase with firmly established active site and subsite features (Table 6.6). It has potential for use as an antimicrobial agent, particularly against Gram-positive microorganisms [141]. It is among the smallest enzymes at 14 kDa and the most common source is hen's egg white. It is stable at slightly acidic pH and loses activity in egg white during storage as pH of egg white rises to $\sim$9. It has been used as an antiseptic agent in cheese manufacture [142] and prevents "late blowing" (gas formation) by *Clostridium* spp. in some cheeses [3,48].

6.3.3 Enzymes Transforming Proteins [141]

Proteinases or proteases are used interchangeably to refer to enzymes that hydrolyze proteins. Rules of nomenclature permit the use of these terms, but the preference is to describe such enzymes as exopeptidases or endopeptidases. Proteases are some of the best-characterized enzymes in recognition of their vital role in the human digestive system and early commercialization (Christian Hansen marketed a standardized calf rennet for cheese making in 1874). Peptidases that transform food proteins *in situ* or are added exogenously to cause protein transformation belong to one of four classes, each of which is described next.

6.3.3.1 Serine Proteases

Among the proteolytic enzymes first studied were the serine proteases secreted by the pancreas, trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and elastase (EC 3.4.21.37), since they were involved in human digestion and nutrient assimilation. The serine protease subtilisin (from *B. subtilus*) was featured as an example of nucleophilic mechanism assisted by a charge-relay system in Figure 6.4.

Most members of this group have molecular masses of 25–35 kDa, and they are characterized by a surface groove or cleft as the substrate binding site. Selectivity is conferred by recognizing either the N-terminal (P_1, as for the pancreatic enzymes listed above) or C-terminal (P'_1) residue comprising the scissile peptide bond. Subtilisins from various *Bacillus* spp. are widely used in the preparation of protein hyrolysates, and they tend to exhibit broad selectivity among amino acids comprising the peptide bond (the S_4/P_4 interaction also impacts selectivity). Pancreatic endopeptidases are also used in various applications and their selectivity patterns were illustrated in Figure 6.14.

6.3.3.2 Aspartic (Acid) Proteases

Aspartic proteases are characterized by two highly conserved ASP residues as the catalytic unit, and most are also active under acidic conditions (pH 1–6) with optima near pH 3–4 [141]. Familiar members of this group include the digestive enzyme pepsin, calf chymosin (also called "rennin" or "rennet," used in cheese-making), cathepsin (which may be involved in postmortem meat tenderization), and the chymosin-substitute peptidases from *Mucor* spp. These endopeptidases are typically 34–40 kDa in mass, monomeric with two protein domains separated by a deep substrate binding pocket. The reaction mechanism for hydrolysis involves a dyad of conserved ASP residues acting as general acid/base ($ASP_{34,216}$ in chymosin) and a noncovalent intermediate (Figure 6.22 [116]). The acidic ASP residue donates an H^+ to activate the peptide C═O, while the ASP carboxylate residue abstracts an H^+ to enhance the nucleophilicity of water. The noncovalent tetrahedral amide hydrate intermediate is rearranged to the zwitter ion by the reversal of roles of the catalytic ASP residues just prior to collapsing to the hydrolysis products and restoring the native enzyme. Aspartic proteases are also known to cause transpeptidation reactions by a mechanism that remains ambiguous.

Selectivity in protein hydrolysis among aspartic proteases is quite similar in that they recognize nonpolar residues (aromatic, LEU) with broad selectivity (including ASP, GLU) at substrate site P_1. An analysis of the unique features of chymosin specificity toward κ-casein analogues was presented earlier (Table 6.4).

6.3.3.3 Cysteine (Sulfhydryl) Proteases [116,141]

Cysteine proteases are a diverse group of enzymes (over 130 known) present in animals, plants, and microorganisms. Most members of this group belong to the papain family, with other members being chymopapain (EC 3.4.22.6) (multiple isoforms) and caricain (EC 3.4.22.30) from the latex of *Carica papaya*; actinidin (EC 3.4.22.14) from kiwi fruit and gooseberry; ficin (EC 3.4.22.3) from fig (latex); bromelain (EC 4.3.22.4) from pineapple; as well as lysosomal cathepsins from animal tissues [141]. A unique cysteine protease system in muscle is calpain (multiple isoforms), a two subunit enzyme that

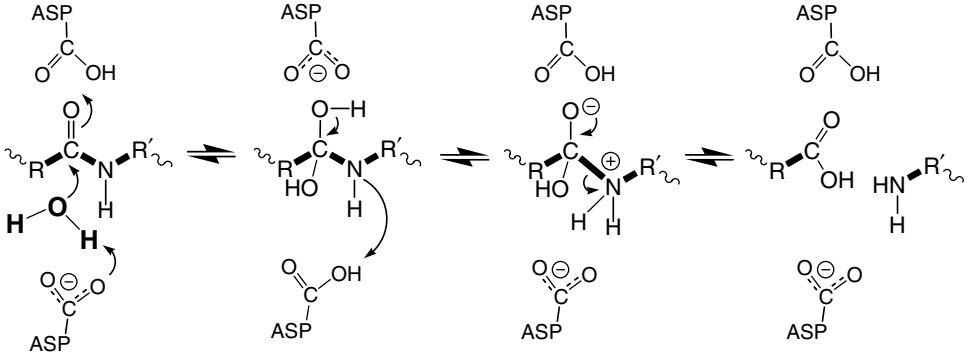


FIGURE 6.22 Reaction mechanism of aspartic proteases. (Redrawn from Sinnott, M. (Ed.) (1998). *Comprehensive Biological Catalysis. A Mechanistic Reference*, Vol. I, Academic Press, San Diego, CA.)

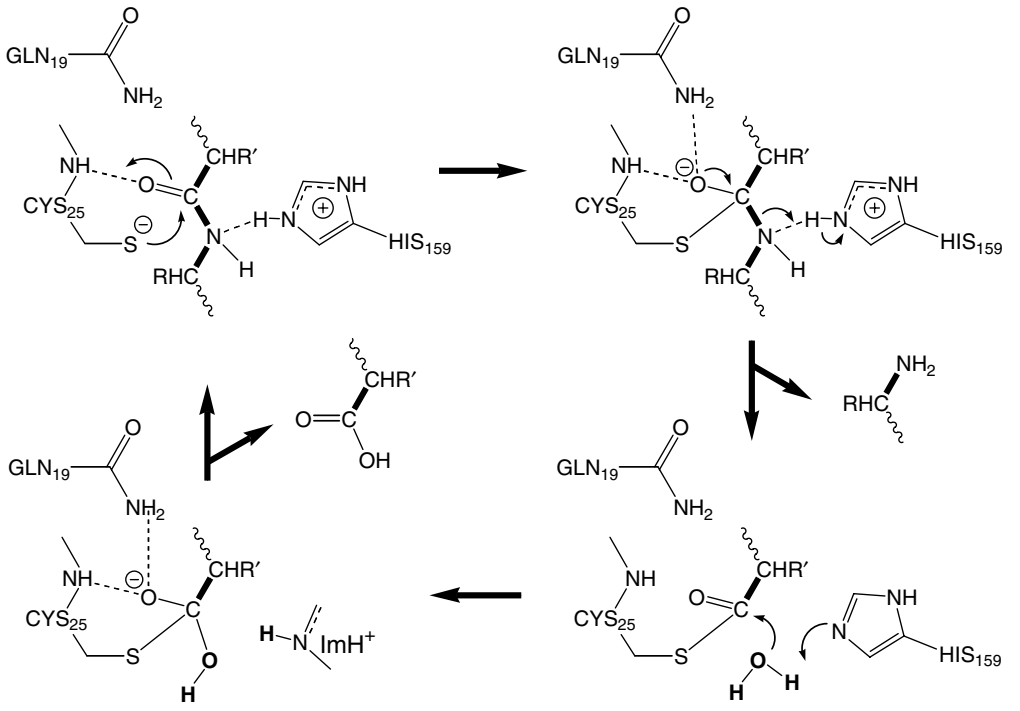


FIGURE 6.23 Reaction mechanism of cysteine proteases. (Redrawn from Sinnott, M. (Ed.) (1998). *Comprehensive Biological Catalysis. A Mechanistic Reference*, Vol. I, Academic Press, San Diego, CA.)

is activated by Ca^{2+} and has a role in postmortem tenderization of muscle. Typically, the enzymes of this group are 24–35 kDa in mass, are optimally active at pH 6.0–7.5, and can withstand temperatures up to 60–80°C (conferred in part by three disulfide bonds). Conserved residues (papain as reference) include the ion-pair catalytic unit formed by CYS_{25} and HIS_{159}, and assisted by ASN_{175}, while GLN_{19} helps to stabilize the oxyanion intermediate. Each of the two protein domains contributes one catalytic residue of the ion pair, positioned at a deep cleft between the domains. The mechanism is unique in that nucleophilic and general-acid catalysis occur through a thiolate–imidazolium ion pair (Figure 6.23 [116]). The thiolate (RS^-) group attacks the electrophilic amide C, yielding a covalent intermediate oxyanion stabilized by the amide NH of CYS_{25} and GLN_{19}. General-acid protonation of the leaving amine group by HIS_{159} yields the thioester intermediate which is ultimately displaced by HIS_{159}-activated water (through another tetrahedral intermediate). Not shown is the role of ASN_{175}, where the amide oxygen H-bonds with the imidazole $N^{\varepsilon 2}$ atom of HIS_{159}. Cysteine proteases are similar in terms of hydrolytic selectivity. They are considered to have broad selectivity for peptide bonds, with a preference for aromatic and basic amino acids at P_1 and nonpolar substrate residues (especially PHE) at P_2 of the peptide substrate (Figure 6.13).

6.3.3.4 Metalloproteases

Metalloproteases constitute the fourth general class of proteolytic enzymes. The most familiar members of this group include exo-acting carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.17.1, a digestive enzyme), endo-acting thermolysin (from *Bacillus thermoproteolyticus*, EC 3.4.24.27) originally isolated from a hot spa in Japan, and the neutral endoprotease from *Bacillus amyloliquefaciens* [141]. Most metalloproteases of relevance to quality and processing of food systems are exo-acting and require Zn^{2+} as the metal. They are classified into five families based on the HIS-rich metal binding motif with a GLU residue, since their primary sequences represent proteins

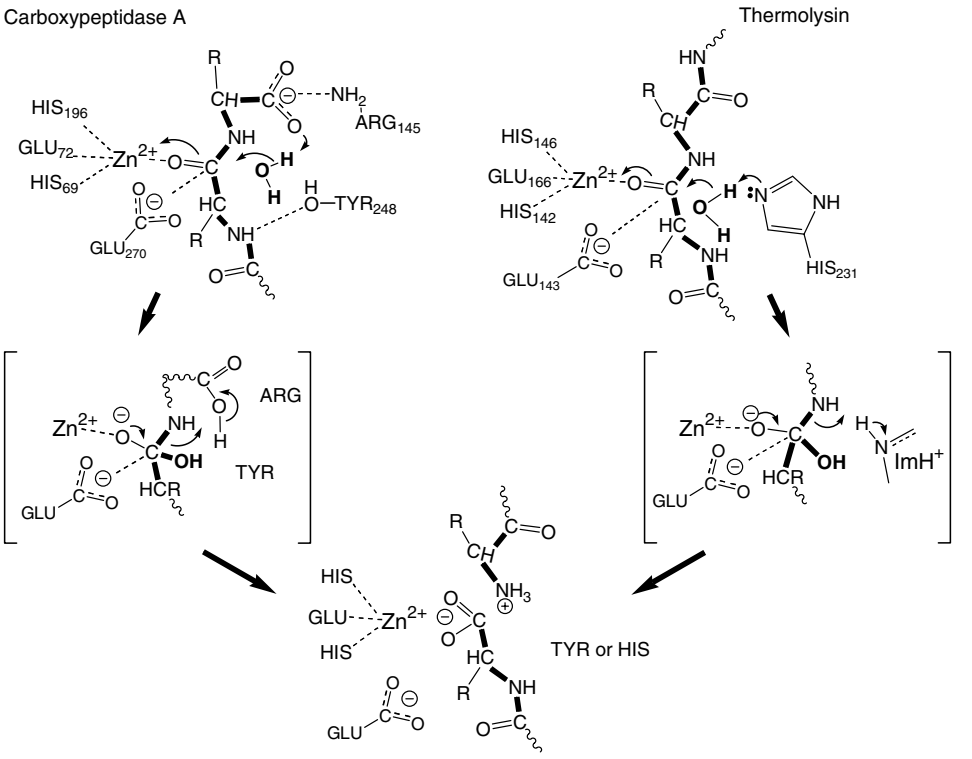


FIGURE 6.24 Reaction mechanism of metalloproteases. (Compiled from Sinnott, M. (Ed.) (1998). *Comprehensive Biological Catalysis. A Mechanistic Reference*, Vol. I, Academic Press, San Diego, CA.)

varying in size from 15 to 87 kDa. Both carboxypeptidase A (87 kDa) and thermolysin (35 kDa) have hydrophobic binding pockets that favor nonpolar and aromatic amino acid side chains (especially LEU, PHE) positioned at substrate subsite P_1'. In carboxypeptidase, a small "hole" is created in part by ARG_{145} and ASN_{144} at enzyme subsite S_1', and this confers the *C*-terminal, exo-acting nature of this enzyme by coordinating with the P_1'—COO^- group. Thermolysin (an endopeptidase) does not have as constrained a binding pocket as carboxypeptidase and can host a longer segment of the peptide.

A unifying mechanistic model has been proposed for metalloproteases, while leaving room for diversity in catalytic residues [116]. For thermolysin, the Zn^{2+} is coordinated with $^-OH/H_2O$ (coordinate has a pK_a of ~5), which is displaced by binding of substrate (Figure 6.24 [116]). HIS_{231} acts as a general base catalyst with a pK_a of ~8 (assisted by ASP_{226}), to activate the nucleophilic water. While GLU_{143} was once thought to be the catalytic base, it is now believed to offer electrostatic stabilization to the δ^+C—O tetrahedral intermediate of the scissile peptide bond; Zn^{2+} also coordinates with the carbonyl δ^-O of the scissile peptide bond. Finally, collapse of the intermediate to yield the product peptides restores the active site. For carboxypeptidase A, the absence of HIS to act as a general base is compensated by the ability of the carboxy terminal residue of the *substrate* to activate nucleophilic water (substrate-assisted catalysis is not rare). Otherwise the mechanistic features are nearly identical to that for thermolysin. Although the exopeptidase action of carboxypeptidase may be conferred by the small hydrophobic binding pocket, that fact that substrate must provide the general base (carboxylate) function may be equally as important.

6.3.3.5 Applications of Proteolytic Action [77,141,142]

Commercial proteases are available at various levels of purity, and some contain multiple proteolytic agents as is typical of *Aspergillus* spp. preparations. Depending on the application, there may be a

need for either strict or broad selectivity in protein hydrolysis. Broad selectivity can also be obtained by adding multiple protease preparations. In many cases, proteases secreted by fermentative organisms, whether adventitious or deliberately added as a culture, contribute substantially to proteolysis in food matrices. Some of the important commercial applications of proteolytic enzymes are described in this section.

6.3.3.5.1 Protein Hydrolysates [77,141]

Hydrolysis of proteins by peptidases is done to improve protein/peptide functionality in terms of nutritional, flavor/sensory, textural and physicochemical (solubility, foaming, emulsifying, gelling) properties, as well as reduced allergenicity (specific examples are cited in Chapter 5). Typically, a protein isolate is treated by a selected endopeptidase as a batch process for a few hours, after which the added enzyme is inactivated by thermal treatment. The most important factors that govern choice of protein as source of the hydrolysate are the value/cost and intrinsic functional properties (which are limited in some fashion to warrant hydrolytic processing), the amino acid composition, and to some extent the primary sequence, if known. These factors are considered in context with the known selectivity of the endopeptidase, especially if there are preferred sites of hydrolysis to obtain the desired functionality. In addition, pH and temperature requirements also impact the suitability of choice among candidate proteins and peptidases to obtain a desired outcome. Endopeptidases are usually employed to attain rapid decreases in average molecular weight of peptides, whereas, exopeptidases are used to hydrolyze small oligopeptides to composite amino acids.

Proteins (commonly meat, milk, fish, wheat, vegetable, legume, and yeast sources) may be subjected to a pretreatment that renders them partially denatured as this enhances peptidase access and hydrolytic attack (excessive denaturation may lead to aggregation and hinder hydrolysis). Protein:enzyme levels are sufficiently high that the enzyme reacts at nearly V_{max} with limited autodigestion of the enzyme, although product inhibition by accumulating peptides may attenuate reactivity. Protein levels in batch reactions are often 8–10%, provided there are no limitations on solubility, and the amount of enzyme added is generally ~2% on a protein basis, depending on purity. Reaction progress is monitored by one of several means (see Chapter 5), and reaction is quenched when the desired degree of hydrolysis (DH) is achieved. Typically, respective DH values of 3–6% (average peptide size of 2–5 kDa) is desired for physicofunctionality, DH ~8+% and 1–2 kDa average peptide size for optimal solubility for use in sports and clinical nutrition products, and more exhaustive DH (as high as 50–70%) to yield small peptides and amino acids of <1 kDa average size for purposes of infant and hypoallergenic foods, and savory flavoring ingredient preparations (soups, gravies, sauces). The greater the DH, the greater the opportunity for bitter peptides (small and hydrophobic) to accumulate, and measures are often required to control this potential flavor defect (discussed later). More recently, there have been reports of preparing bioactive peptides from protein hydrolysates, including Ca^{2+}-binding phosphopeptides from casein for enhanced mineral bioavailability, antioxidant preparations, and peptides that inhibit angiotensin-converting enzyme in human plasma (as a potential intervention to lower blood pressure). Proteases can also be used to isolate residual muscle protein from bones of fish and land animals as protein hydrolysates, and this usually involves incubation at 55–65°C for 3–4 h. The final product mixture from enzyme hydrolysis of proteins may require posttreatment refining and/or separation to obtain a product derivative ideally suited for the intended application.

6.3.3.5.2 Milk Clotting [3,141]

Calf chymosin (rennet) and chymosin substitutes are added to milk to cause the initial milk-clotting reaction leading to cheese manufacture. Milk-clotting activity is related to the specific hydrolysis of the PHE_{105}–MET_{106} bond of κ-casein, liberating a glycomacropeptide (the enzymic step) that creates a hydrophobic surface on the micelles prompting them to aggregate (a nonenzymic step). The unique selectivity of chymosin was featured earlier (Table 6.4). Starter cultures are added to milk at 40–45°C to cause a pH decline to 5.8–6.5, upon which chymosin is added to initiate clotting. As a

result of subsequent steps of cheese manufacture, some enzyme activity remains in the curd and contributes to cheese ripening and flavor development during aging. Proteases in the starter cultures also contribute to sustained proteolysis and flavor development during aging. A recombinant chymosin from *E. coli* K-12 was the first genetically engineered enzyme to be approved for use in foods and similar commercial preparations are in widespread use. Chymosin substitutes include bovine and porcine pepsins, and aspartic endopeptidases from *Rhizomucor* spp. and *Cryphonectria parasitica*, which possess progressively reduced milk clotting:proteolytic activities ratios in the order listed (which leads to reduced process yield and potential bitterness in cheese).

6.3.3.5.3 Meat Tenderization [3,127]

Papain and other sulfhydryl endopeptidases (bromelain and ficin) are applied to muscle or meats that do not become sufficiently tenderized during postmortem aging. These enzymes are effective in this application because they can hydrolyze collagen and elastin, connective tissue proteins that cause toughness in meat. However, the two drawbacks of tenderization by exogenous endopeptidases are that they can be "overdosed" and the pattern of tenderization is not the same as that which occurs in naturally aged/tenderized meat (proteolysis selectivity patterns are different). Enzyme (usually papain) in a powdered form (using salt or other innocuous material as carrier) can be applied directly to the surface of meats, or the enzyme in dilute saline can be injected or applied as a dip. Antemortem application of enzyme is possible, as a fairly pure solution in saline injected intravenously into animals 2–10 min before slaughter, sometimes after stunning; this helps distribute the enzyme throughout the muscle tissues. Injection of inactivated papain (disulfide form) obviates any discomfort among animals, since the enzyme becomes activated by the reducing conditions that soon prevail postmortem. In many cases, owing to the relative thermal stability of these endopeptidases, perhaps as much tenderizing effect occurs during the cooking phase of meat preparation as it does during chilled handling and storage of meat.

6.3.3.5.4 Beverage Processing [77,127]

In beer, a defect referred to as chill-haze may be caused by the association (complexing) of tannins and proteins in beer. Papain has long been used (since 1911) to hydrolyze protein and minimize haze formation, although bromelain and ficin as well as other bacterial and fungal proteases may now be used for this purpose. The endopeptidase is added postfermentation and prior to final filtering. Papain is ultimately destroyed by typical beer pasteurization, and excessive action of papain may lead to loss in foam stability [142]. Other proteases, particularly the *B. amyloliquefaciens* neutral protease, are added during the mashing step to increase soluble nitrogen from protein to support subsequent fermentation (it would also leave less protein to participate in haze formation). Controlled or measured proteolysis in beer is important, since some residual protein is necessary to maintain specific quality attributes.

6.3.3.5.5 Dough Conditioning [3,77,142]

Dough formulations and various types of flour (bread or biscuit quality) confer the strength and rheological properties of the dough and this impacts final product quality. Dough pH is usually ~6.0, but may range widely and approach pH 8.0 in a few cases; available proteases have pH optima matched to the alkaline pH range with bacterial (*Bacillus* spp.) enzymes, and toward the acidic range with fungal (*Aspergillus* spp.) enzymes. Proteases are used to modify and optimize dough strength for a particular product, and serve to reduce mixing time to obtain the proper dough viscoelasticity. Proteases can also improve the performance of flours with damaged gluten that confer less elastic and stiffer doughs. Exogenous proteases are added to affect controlled hydrolysis of the gluten during the dough conditioning stage, although proteases may continue to act during baking until thermally deactivated. Hydrolysis of gluten weakens the gluten network, resulting in enhanced extensibility and viscoelasticity of the developed dough, and these properties are associated with increased bread volume, uniform crumb development, and tenderness of the final product. Controlled hydrolysis is obtained by making use of a protease with moderate peptide bond selectivity (to guard against

TABLE 6.7
Hydrolytic Selectivity of Proteases Toward Major Gluten Proteins

Protease Preparation	Relative Activity toward		Ratio of Activity on Glutenin:Gliadin
	Glutenin	Gliadin	
A	1.00	2.17	0.46
B	0.50	0.17	3.0
C	0.69	0.064	11
D	1.30	0.90	1.4
E	0.37	0.19	2.0
F	0.55	0.87	0.63
H	2.07	3.02	0.68
I	2.68	0.38	7.0
G	0.60	0.038	16

Note: Individual protease preparations were not specified in the original survey.

Source: Adapted from Tucker, G.A. and Woods, L.F.J. (Eds.) (1995). *Enzymes in Food Processing*, 2nd edn., Blackie, New York.

exhaustive hydrolysis), and dosing at a rate that provides the desired degree of hydrolysis prior to deactivation during the baking cycle. Excessive proteolysis will yield low product volume and textural defects. Use of less specific proteases (or mixtures of proteases) is appropriate when weaker doughs are required for forming into shapes, such as pizza crust, wafers, or biscuits. Choice of protease can be critical to product quality since proteases have different specificities of reaction with the major gluten proteins, either gliadin or glutenin (Table 6.7 [127]). Such differences can account for different degrees of performance enhancement of doughs by choice of exogenous protease.

6.3.3.5.6 Flavor Modulation (Debittering) [97]

Protein hydrolysates and fermented foods subjected to intermediate degrees of proteolysis by endoproteases (cheese, cocoa, beer, cured meats, fish sauce, soy) may develop bitterness when small hydrophobic peptides accumulate beyond taste thresholds. Exopeptidases are used to "debitter" such foods and are available from bacterial, fungal, and plant and animal sources (over 70 are catalogued by IUBMB). Exopeptidases may be specific for the C-terminus (carboxypeptidases) or N-terminus (aminopeptidases), and may be specific for liberating a single amino acid, dipeptide or tripeptide from the substrate. Superimposed on these types of specificity is selectivity for some over other amino acid residues at the substrate P_1/P'_1 or P_2/P'_2 site(s), examples being X-PRO-dipeptidyl aminopeptidase and LEU-aminopeptidase. Exopeptidases often require the action of prior and specific endopeptidases to ensure that bitter peptides are efficiently degraded. Exopeptidases from lactic acid bacteria are among the best characterized, and such an understanding allows the strategic use of fermentative or starter cultures, or cell-free extracts to control bitterness in fermented or proteolyzed foods. As an example, *Lactobacillus helveticus* CNRZ32 is a commercial strain that reduces bitterness and intensifies flavor development in cheese. It has a complex proteolytic enzyme system that includes endopeptidases with postproline specificity (PRO at S_1) and a general aminopeptidase [15]. Acting in concert, these enzyme activities facilitate that bitter peptides being degraded to free amino acids, thereby reducing bitterness.

6.3.3.5.7 Aspartame Synthesis [16,58,141]

Thermolysin, a metalloprotease, is used to synthesize aspartame (L–ASP–L–PHE—OCH_3), a sugar substitute used primarily in low-calorie soft drinks. Thermolysin is a catalyst especially suited for this process: it is stable to 90°C, while being optimally active at 80°C. It is activated more than tenfold

by high salt levels (1–5 M) allowing it to function well at high osmolarities (high [substrate]); it tolerates organic solvents, is selective for preparing the peptide bond at the α-COOH group of ASP (the chemical method can cause reactions at the β-COOH group of ASP, creating a bitter analogue), and does not hydrolyze the methyl ester group of PHE (which is required for sweetening). The enzyme synthesis process has evolved to use an immobilized thermolysin in a batch reactor with monophasic ethyl acetate–water as reaction medium, affording yields of >95% at 55°C.

6.3.3.6 Transglutaminase [35,141]

Transglutaminases (TGs) (EC 2.3.2.12, γ-glutamyl-peptide, amine-γ-glutamyl-transferase) occur in animals, plants, and microorganisms (especially *Steptoverticillium* spp.). In animals they have critical roles in fibrin cross-linking (blood clotting) and keratinization (epidermal tissue development) among other functions; in plants they appear to be involved in cytoskeleton and cell wall formation, while in bacteria they may be involved in coat assembly in sporulating cells. Mammalian TGs are typically monomeric proteins of 75–90 kDa, while microbial enzymes are about 28–30 kDa. They typically require Ca^{2+} for activity, and have neutral to slightly alkaline pH optima. As with endopeptidases, partially denatured or unfolded proteins provide improved access of TG. The types of reactions TGs catalyze are (⊩ represents the protein backbone)

$$\text{Cross-linking: } \Vdash\text{GLN}-\text{CO}-\text{NH}_2 + \text{NH}_2-\text{LYS}\dashv \rightarrow \Vdash\text{GLN}-\text{CO}-\text{NH}-\text{LYS}\dashv + \text{NH}_3 \quad (6.26)$$

$$\text{Acyltransfer: } \Vdash\text{GLN}-\text{CO}-\text{NH}_2 + \text{NH}_2-\text{R} \rightarrow \Vdash\text{GLN}-\text{CO}-\text{NH}-\text{R} + \text{NH}_3 \quad (6.27)$$

$$\text{Deamidation: } \Vdash\text{GLN}-\text{CO}-\text{NH}_2 + \text{H}_2\text{O} \rightarrow \Vdash\text{GLN}-\text{COOH} + \text{NH}_3 \quad (6.28)$$

These reactions provide the basis for applications in foods. The most important reaction is cross-linking of proteins by an isopeptide bond (Equation 6.26) that has the capacity to increase the size of the resulting proteins and create a vast network within the food matrix. Examples where this is exploited are the creation of irreversible and temperature stable gels by cross-linking egg, milk, or soy proteins and gelatin.

Addition of TG during the early stages of yogurt production serves to increase gel strength and reduce syneresis, while in cheese manufacture it may provide greater yield of protein. In baked goods, addition of TG to dough facilitates the formation of a gluten network, enhancing dough stability, gluten strength, and viscoelasticity, leading to improved volume, structure, and crumb of the final product. For muscle foods, applications of TG revolve around enhancing or controlling the gel strength of surimi products, and to serve as a binding agent for the creation of formed meat products from low-value small or minced meat fragments, as well as enhancing protein gel strength of ham and sausage products.

6.3.4 Lipid-Transforming Enzymes

6.3.4.1 Lipase

Lipases (EC 3.1.1.3; triacylglycerol acylhydrolase) are distinct from other carboxylesterases in that they act only at the oil–water interface. This requirement is easily seen in the relationship between rate of reaction and increasing levels of substrate (Figure 6.25 [107]). While esterases react with soluble substrates by conventional Michaelis–Menten kinetics, lipases do not readily access substrate until it has exceeded its solubility and starts to form colloidal aggregates, such as micelles, that pose an interface. Lipases and carboxylesterases almost invariably possess the catalytic triad of GLU(ASP)–SER–HIS as the transforming locus as illustrated for serine proteases (Figure 6.4). Thus, the mechanism of acyl–enzyme intermediate and two tetrahedral intermediates applies to lipases as well.

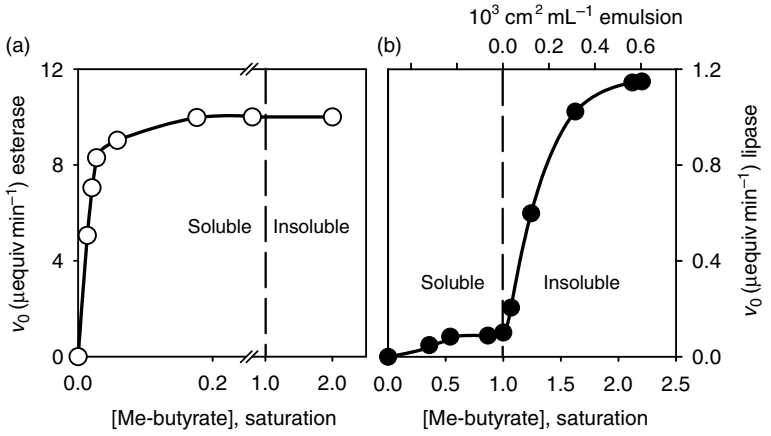


FIGURE 6.25 Differentiation between (a) esterase and (b) lipase on the basis of substrate properties. (Redrawn from Sarda, L. and Desnuelle, P. (1958). *Biochim. Biophys. Acta* 30:513–520.)

While activity of endogenous lipases is often associated with acylglycerol hydrolysis and problems with lipid degradation and/or hydrolytic rancidity (or leading to oxidative rancidity since liberated fatty acids tend to be more prone to oxidation), exogenous lipases are used for beneficial purposes. Currently, commercial uses of lipases involve liberating flavoring (short-chain) fatty acids from lipids and rearranging fatty acyl groups along the glycerol backbone to create highly valued and functional triacylglycerols from low-value lipids. Both of these applications are founded on selecting lipases with reaction selectivities required to yield the desired products.

Selectivity of lipases was introduced in Figure 6.16, and involves selectivity toward fatty acyl group, ester position along the *sn*-glycerol backbone, size of the glyceride (mono-, di-, or triacylated), as well as interactions among these factors, which confer characteristic stereoselectivity. The types of selectivity exhibited by many of the commercially relevant or promising lipases of well over 100 characterized sources are shown in Table 6.8 [2,51,69,89,92,94,99,123,146]. Particularly rare types of selectivity include the preference toward *sn*-2-glycerol sites exhibited by *Candida antarctica* A lipase and a minor lipase isoform of *Geotricum candidum*, although this feature may be linked to the type of substrates (fatty acyl groups) used in studying this trait. Many lipases in Table 6.8 have been analyzed for stereoselectivity (Figure 6.16d). Lipases typically have optimal pH and temperature ranges of 5.0–7.0°C and 30–60°C.

6.3.4.2 Lipase Applications

6.3.4.2.1 Flavor Generation

Lipases used to generate aged-related "piccante" flavors in cheeses, especially of the Italian and mold-ripened varieties, are selective for hydrolyzing short-chain (C4—C8) fatty acids from triacylglycerols of milk fat, and include pregastric lipases from goat, lamb, and calf [3,48,142]. Since these short-chain fatty acids are enriched at the *sn*-3-glycerol position, a lipase that is selective for this site would also be applicable for this purpose. The lipase in papaya latex is selective for the *sn*-3-glycerol position, but since papaya latex contains papain, it would not be suitable in cheese. Alternatively, some microbial lipases (*C. rugosa*, or *R. miehei* and *A. niger*) are also known to release short-chain and/or *sn*-1,3-linked fatty acids from milk fat (Table 6.8). Most lipases hydrolyze unsaturated fatty acids that may be precursors to oxidative products of ketones and lactones, some resulting from microbial metabolism. Lipases are also used to prepare enzyme-modified cheese for use as processed cheese, spreads, sauces, or flavoring ingredients, and subsequent pasteurization serves to destroy residual enzyme activity. Overdosing of enzyme can lead to soapy or overly pungent flavor.

TABLE 6.8
Selectivity Patterns of Some Lipases of Commercial Interest or Used in Commercial Applications

Lipase	Preferences toward			Other Feature or Comment
	sn-Glycerol Sites	Fatty Acid[a]	Glycerolipid[b]	
1. *Aspergillus niger*	*sn*-1,3 ≫ *sn*-2	Short-chain, 16	AG	
2. *Candida antarctica* A and B forms	A: *sn*-2 > *sn*-1,3	Short-chain, 18:*X*	AG	
	B: *sn*-1,3 > *sn*-2	6–10 > broad	AG; GL	Fatty acid binding pocket ~13C
3. *Candida rugosa*	*sn*-1,3 > *sn*-2; nonspecific	4,8 > broad	AG	Multiple isoforms (formerly *C. cylindraceae*) Fatty acid binding pocket ~17C
4. *Carica papaya*	*sn*-1,3 > *sn*-2	4, short-chain	AG	Latex source contains papain
5. Geotricum candidum	Nonspecific; *sn*-2 > *sn*-1,3	8, long-chain, 18:*X*	AG	Multiple isoforms (minor isoform is *sn*-2 selective)
6. Patatin (potato tuber)	*sn*-1,3 > *sn*-2	8,10	MAG > DAG; GL, PL	General lipid acyl hydrolase
7. *Penicillium* spp.	Nonspecific; *sn*-1,3 > *sn*-2	Long-chain	MAG, DAG	Multiple isoforms
8. Pancreatic	*sn*-1,3 (strictly specific)	4 > broad	AG	Fatty acid binding pocket ~8C
9. *Pseudomonas* spp.	Nonspecific; *sn*-1,3 > *sn*-2	8,16	AG	*Burkholderia* spp. similar fatty acid binding pocket ~14C
10. *Rhizomucor miehei*	*sn*-1,3 ≫ *sn*-2	8–18	AG; PL, GL	fatty acid binding pocket ~18C
11. *Rhizopus arrhizus*	*sn*-1,3 ≫ *sn*-2	8–14	AG; GL, PL	*Rhizopus* spp. lipases almost identical

[a] Fatty acids are designated as number of carbons in *n*-acyl chain; 18:*X* denotes 18C fatty acid with $X = 0$–3 double bonds.

[b] AG = acylglycerols; GL = glycolipid; PL = phospholipid; MAG = monoacylglycerol; DAG = diacylglycerol. Ambiguities and inconsistencies among compiled observations are common and are founded on the variety of reaction designs in which selectivity patterns are established.

Source: Ader, U., et al. (1997). In *Methods in Enzymology*, Rubin, B. and Dennis, E.A. (Eds.), Vol. 286, *Lipases, Part B. Enzyme Characterization and Utilization*, Academic Press, New York, pp. 351–387; Gunstone, F.D. (Ed.) (1999). *Lipid Synthesis and Manufacture*, CRC Press LLC, Boca Raton, FL, p. 472; Lee, C.-H. and Parkin, K.L. (2001). *Biotechnol. Bioeng.* 75:219–227; Persson, M., et al. (2000). *Chem. Phys. Lipids* 104:13–21; Pinsirodom, P. and Parkin, K.L. (2000). *J. Agric. Food Chem.* 48:155–160; Pleiss, J., et al. (1998). *Chem. Phys. Lipids* 93:67–80; Rangheard, M.-S., et al. (1989). *Biochem. Biophys. Acta* 1004:20–28; Sugihara, A., et al. (1994). *Prot. Eng.* 7:585–588; and Yamaguchi, S. and Mase, T. (1991). *Appl. Microbiol. Biotechnol.* 34:720–725.

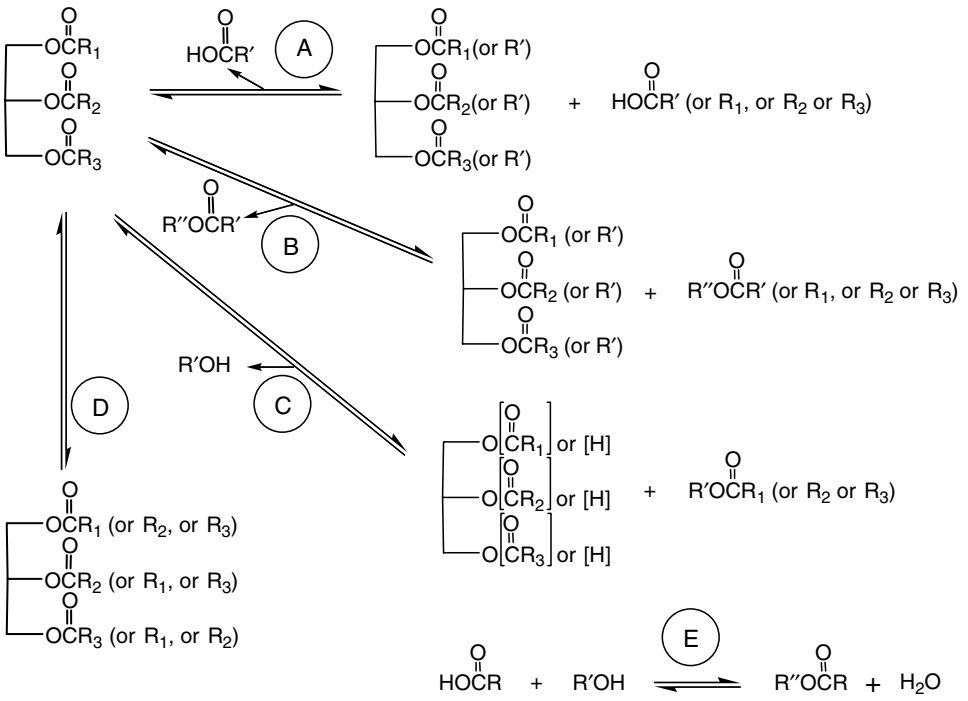


FIGURE 6.26 Types of acyl-restructuring reactions mediated by lipase in microaqueous media. (A) Acidolysis, (B) transesterification, (C) alcoholysis, (D) interesterification, and (E) esterification.

6.3.4.2.2 Acylglycerol Restructuring

Another major use of lipases is for the strategic rearrangement of fatty acyl groups to yield a predetermined distribution along *sn*-glycerol to create high-value lipids from low-value ones [51]. The intended result is the preparation of "structured lipids." The basic approach to lipase restructuring of lipids is the use of microaqueous (<1% moisture) reaction media comprised primarily of organic solvent or just lipid substrate itself (which can serve as "solvent"). Under these conditions, net reactivity of lipids with the lipase is in the direction of ester (re)synthesis and not hydrolysis. The various types of lipase-mediated processes that can be conducted (Figure 6.26) involve reactions of a single triacylglycerol substrate alone (interesterification, path D); between a triacylglycerol substrate and an exogenous source of fatty acid(s) (acidolysis, path A), fatty acyl ester(s) (transesterification, path B) or alcohol(s) (alcoholysis, path C); or between fatty acid and alcohol cosubstrates (esterification, path E). The most successful applications have strategically exploited the characteristic selectivities of lipases combined with the known distribution of fatty acids within the starting material (natural sources of triacylglyerol), and such applications will be highlighted in the next paragraph.

Cocoa butter is a premium fat because of its high natural "purity" with >80–90% of the triacylglycerol molecular species being POSt (38–44%), StOSt (28–31%), and POP (15–18%),* providing a sharp, cooperative melting profile ([51], Chapter 4). Cocoa butter substitutes can be prepared using an *sn*-1,3-regioselective lipase and a palm oil midfraction (58% POSt) combined with exogenous stearic acid using an "acidolysis" approach (Figure 6.26a) in a stirred-tank reactor for 16 h at 40°C. The result is a product that is 32% POSt, 13% StOSt, and 19% POP. The process makes use of *Aspergillus*, *Rhizomucor*, or *Rhizopus* lipases, which can also be immobilized in a packed bed reactor for faster

* Triacyl-*sn*-glycerol species are identified using the shorthand designations of fatty acids (Chapter 4) of St for stearic acid, P for palmitic acid, O for oleic acid, listed in order as occurring at the *sn*-1, *sn*-2, and *sn*-3 positions.

product throughput. Of more recent commercialization is the preparation referred to as "Betapol," a fat derivative enriched in POP, which is the major triacylglycerol in human breast milk [111]. Thus, POP comprises a nutritional product for use in infant formula. In this application tripalmitin (PPP; enriched in palm stearin) is a suitable starting material, and can be reacted with oleic acid (1:1 w/w) in an acidolysis reaction (Figure 6.26a) with an *sn*-1,3-selective lipase. A two-stage process with an *sn*-1,3-selective lipase involves an initial alcoholysis reaction of PPP with ethanol (Figure 6.26c) to yield an *sn*-2-palmitoylglycerol, followed by an esterification reaction (Figure 6.26e) in the presence of oleic acid. Betapol can also be prepared from native lipid resources of PPP-rich palm oil fraction and high-oleic sunflower or canola oils. Similar approaches can be used to prepare other "structured lipids" with lipases, including medical/dietetic lipids, but these have not been widely adopted commercially at this writing.

6.3.4.2.3 Dough Improvement

Lipases are common ingredients in bread doughs [3,127,142]. They supplement endogenous cereal grain lipases and are added as dough improvers, which manifests as increased bread volume, more uniform crumb and air cell size, and lesser tendency to stale, without influencing rheological (mixing) properties of the dough. These improvements derive from lipase hydrolysis of cereal and/or added lipids, giving rise to emulsifying agents, mono- and diacylglycerolipids, which can help incorporate and stabilize small air cells in the dough. Monoacylglycerols can also form inclusion complexes with amylose, and this reduces the tendency for starch to retrograde (stale) after baking. Also the addition of lipases instead of added emulsifiers as ingredients provides a "cleaner" label declaration. Lipases commonly used in baking [48] are sourced from *Rhizomucor* and *Rhizopus* spp., which can hydrolyze glycolipids and phospholipids in addition to acylglycerols (Table 6.8); lyso-phospholipids and lyso-glycolipids are potent surface-active agents. Lipases are also used in noodle formulations as it improves whiteness, an important quality attribute [142]. This effect may result from oxidation of liberated unsaturated fatty acids and bleaching of dough through secondary reactions. Lipase addition also reduces cracking in dried noodles and stickiness upon cooking; this is associated with reduced leakage of starch, perhaps through complexing with fatty acids and lysoglycerolipids.

6.3.4.3 Lipoxygenases

Lipoxygenase action is generally considered to have detrimental effects on food and lipid quality, and this aspect will be addressed later in this chapter. One beneficial use of lipoxygenase is to provide oxidizing power during dough conditioning [142]. Lipoxygenase oxidizes unsaturated fatty acids (made available by added lipases) generating oxidizing conditions that help strengthen the gluten network by affecting disulfide cross-links within the gluten, enhancing dough viscoelasticity. The addition of soy (or bean) flour to bread dough is the preferred way of incorporating lipoxygenase and this can lessen or eliminate the need of more conventional oxidizing agents such as bromates. Secondary oxidation reactions can also destroy endogenous carotenoids and affect bleaching or whitening of the final products, as desired in noodles and some breads.

6.3.4.4 Phospholipases

Phospholipases are classified as types A_1, A_2, C, and D, each with different and exclusive bond selectivities toward phospholipids (Figure 6.27). One commercial application is the addition of phospholipase A_2 (EC 3.1.1.4) (*Aspergillus* spp. and pancreatic sources are common) to crude oil during the degumming stage to hydrolyze phospholipids at the *sn*-2 site to create the corresponding lysophospholipid [48]. This is important for the removal of otherwise nonhydratable phospholipids. Phospholipase A_2 has potential use as an agent to create superior lysophospholipid emulsifiers from phospholipid-rich sources, such as egg yolk [3], and this effect may occur *in situ* in bread manufacture by virtue of addition of lipase with phospholipase A_2-like activities (Table 6.8).

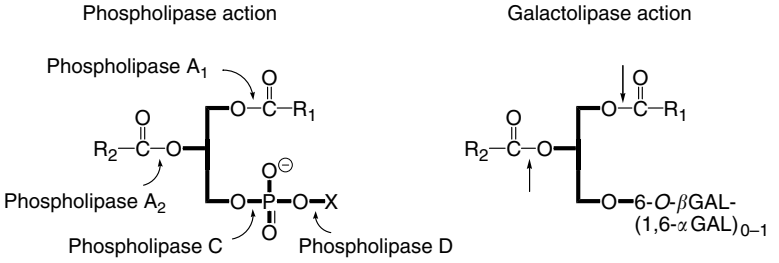


FIGURE 6.27 Bond specificity for lipolytic enzymes acting on polar glycerolipids.

6.3.5 Miscellaneous Enzyme Applications

An acid urease (EC 3.5.1.5, urea aminohydrolase) from *Lactobacillus fermentum* is approved for use in wine to prevent accumulation of urea, which can otherwise react with ethanol to form ethylcarbamate, an animal carcinogen. Hexose oxidase (EC 1.1.3.5) has been added to bread dough where multiple hexoses exist and are available as substrate to yield oxidizing equivalents as dough conditioners [3]. Catalase (EC 1.11.1.6, H_2O_2:H_2O_2 oxidoreductase) is specifically added to remove residual H_2O_2 in milk that has been treated with such to reduce microbial loads when refrigeration is not readily accessible [48]. Sulfhydryl oxidase (formerly EC 1.8.3 unassigned, now considered as thiol oxidase, EC 1.8.3.2, thiol:O_2 oxidoreductase) has long been considered as a solution to cooked flavor defect in UHT milk that is caused by thiols formed during processing [141]. Sulfhydryl (thiol) oxidase from *A. niger* has been suggested as possible dough conditioning agent by providing oxidizing power and forming disulfide bonds in gluten [3].

Going forward, as cost of enzyme production is reduced by biotechnological and genetic advances, enhanced competitiveness of enzyme-mediated processes will lead to expanded commercial uses. Space constraints preclude mention and discussion of other enzymes with commercial potential as processing aids. Enhancing range of thermal and pH stabilities will continue as a priority, and recovery of valuables from agricultural waste streams by enzyme processes is likely to attract expanded attention.

6.4 ENVIRONMENTAL INFLUENCE ON ENZYME ACTION

Temperature, pH, and water activity are among the most important environmental factors that influence enzyme activity, and changes in these parameters comprise the principal physical means to control enzyme action in food matrices. This section will examine the basis for how these factors affect enzyme function.

6.4.1 Temperature

6.4.1.1 General Responses of Enzyme Action to Temperature

Temperature has predictable and opposing effects (activation and deactivation) on enzyme activity. Increasing temperature increases free energy in the system; the net result is the lowering of the energy barrier for reactions to occur and they are accelerated. Recall Equation 6.1 (Section 6.2.3.1), and if the Arrhenius frequency factor "*A*" is substituted for the combined constants "*PZ*," the log transformation yields

$$\ln k = \ln A - \frac{E_a}{RT} \tag{6.29}$$

Equation 6.29 predicts a linear relationship between ln k and $1/T$ with a slope of $-E_a/R$. Greater E_a values signify greater temperature *dependence* of reactions.

Note that this relationship (Equation 6.29) holds only for examining and predicting rate constants (k_x), or parameters composed of, or directly proportional to, rate constants such as k_{cat}, V_{max}, K_M, V_{max}/K_M, K_S, and so forth, provided that reaction order does not change with temperature. Simply measuring enzyme activity under a specified condition does not satisfy this requirement. "Breaks" or discontinuities in the linear (negatively sloped) portion or nonlinearity of Arrhenius plots have been offered as evidence of major biochemical events, such as lipid phase transitions for membrane enzymes or the presence of multiple enzyme isoforms. It is just as likely that such breaks represent a temperature-dependent shift in the magnitude of a rate constant such as K_M, or a change in reaction order, rate-limiting step, or ionization of a critical unit [52,115].

The utility of the Arrhenius plot is that it provides for an estimate of E_a, which is an indicator of catalytic power for an enzyme reaction relative to a corresponding uncatalyzed or chemically catalyzed reaction (cf., Table 6.1). A departure from linearity (but not a "break") on Arrhenius plots for enzyme activity occurs at progressively elevated temperature (at $\sim$0.0030 K^{-1} on the x-axis in Figure 6.28a [6,67]) because of the second effect of temperature on enzymes, which is to cause denaturation. Continuing increases in temperature beyond the maximum or "optimum" for enzyme activity leads to a sharp decline in reaction rate constant, and this positively sloped linear portion of the plot represents an E_a for enzyme deactivation (102 kcal mol^{-1} in this example). E_a values for enzyme deactivation typically range 40–200 kcal mol^{-1} compared to 6–15 kcal mol^{-1} for activation. Protein denaturation involves the unfolding of large segments of the polypeptide chain (a global process), a process requiring greater free energy change than that required for stabilization of the transition state at the active site (a localized process).

It can be difficult to accurately determine reaction v_0 (i.e., linear rates) at temperatures where the enzyme is initially active but rapidly inactivating, as a means to determine the thermal deactivation of enzyme (as in Figure 6.28a). A more direct way of determining the parameters of thermal inactivation of an enzyme is to incubate the enzyme at various temperatures, and test for residual activity remaining under standardized conditions of enzyme assay (usually at optimum pH and a nondeactivating temperature) after various time intervals (Figure 6.28b). The assay for enzyme should use

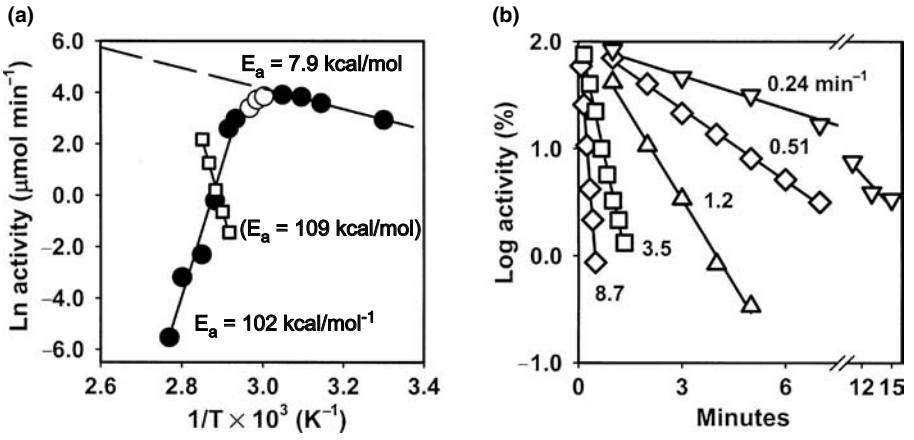


FIGURE 6.28 Thermal sensitivity of tomato fruit pectin methyl esterase. (a) Arrhenius plot (Redrawn from Laratta, B., et al. (1995). *Proc. Biochem.* 30:251–259.), where original data appears as circles and only closed circles were used to construct linear approximations. Open square plot is from data derived from panel (b). (b) First-order deactivation plots (Redrawn from Anthon, G.E., et al. (2002). *J. Agric. Food Chem.* 50:6153–6159.), where increasing slopes of plots correspond to incubation temperatures of 69.8°C, 71.8°C, 73.8°C, 75.8°C, and 77.8°C.

$[S] \gg K_M$,* such that resulting reaction rates are $\sim V_{max}$ ($\propto E_T$) and rate limiting and linear with respect to [E]. Since enzyme deactivation is often a first-order process ($[E_0]$ is the initial level):

$$[E] = [E_0]e^{-kt} \quad \text{and} \quad \ln \frac{[E]}{[E_0]} = -k_d t \tag{6.30}$$

Results are interpreted as semilog plots (a factor of 2.303 is used to interconvert log and ln plots), and for each temperature assessed, a corresponding k_d (deactivation rate constant) can be estimated by linear regression (slopes $= -k_d/2.303$) (Figure 6.28b). The collection of k_d values can be transposed to an Arrhenius plot (Figure 6.28a) to estimate E_a for enzyme inactivation, which is E_a of 109 kcal mol^{-1} in this example. Thus, good agreement is observed from independent studies using alternative means to determine the thermal sensitivity of pectin methyl esterase of tomato fruit.

6.4.1.2 Optimum Temperature for Enzyme Function

A temperature optimum for enzyme activity results from the net activating and deactivating effects of temperature. While the temperature optimum is where enzyme reaction rate (v_0) is greatest, this condition lasts for a limited duration, and, over time, progressive denaturation soon dominates and much of the original activity is lost. An example of typical patterns of thermal behavior of enzymes is provided by pullulanase from *Aerobacter aerogenes* (Figure 6.29a [48,129]). Note the more gentle progression of upward slope for the activity curve at 10–40°C compared to the sharp ascent in deactivation rate constant (k_d) at 50–60°C, and sharp descent in enzyme activity/stability at 50–60°C. These trends of greater thermal dependence (greater E_a values) of enzyme deactivation over temperature activation of reaction can also be seen from the plots for tomato pectin methyl esterase (Figure 6.28a). Thus, as temperature increases, the acceleration of enzyme deactivation at some point becomes the dominant influence of temperature. The temperature-dependent activity and stability profiles of several food-related enzymes are provided in Figure 6.29b. The practical upper temperature limits of an enzyme reaction in food applications are often 5–20°C below the temperature where maximum reaction rate is observed, with the goal to maintain elevated and persistent enzyme activity during the scheduled process.

An analogous plot is reserved for evaluating the temperature influence on equilibrium processes. The plot is similar to Figure 6.28a except that the ordinate is log K, and the slope is proportional to $\Delta H°$, instead of E_a.

$$\frac{d \ln K}{d(1/T)} = \frac{-\Delta H°}{R} \tag{6.31}$$

An example summarizes the temperature dependence of the equilibrium constant (K_{eq}) for the glucose $\rightleftarrows$ fructose isomerization catalyzed by xylose isomerase (Figure 6.30 [98]). This plot finds utility in characterizing temperature dependencies of other equilibria related to optimum enzyme functioning such as $K^{\ddagger}$ for the transition-state theory, ionization of amino acid side chains (K_a) involved in enzyme activity, or enzyme kinetic functions that represent (pseudo-)equilibria (K_M, K_S).

There are other temperature effects on enzyme activity. Cold-deactivation of enzymes may occur for oligomeric enzymes when nonpolar forces are involved in polypeptide association. Low temperature reduces the strength of these interactions (Chapter 5) and may promote dissociation of subunits and compromise activity. Elevated temperature generally reduces aqueous solubility of gases, and reactions that require O_2 may become limiting depending on the K_M for and solubility of dissolved O_2. Some lipid substrates undergo phase transitions over temperature ranges relevant

* Sometimes limits in S solubility or other complicating factor renders this condition difficult to attain.

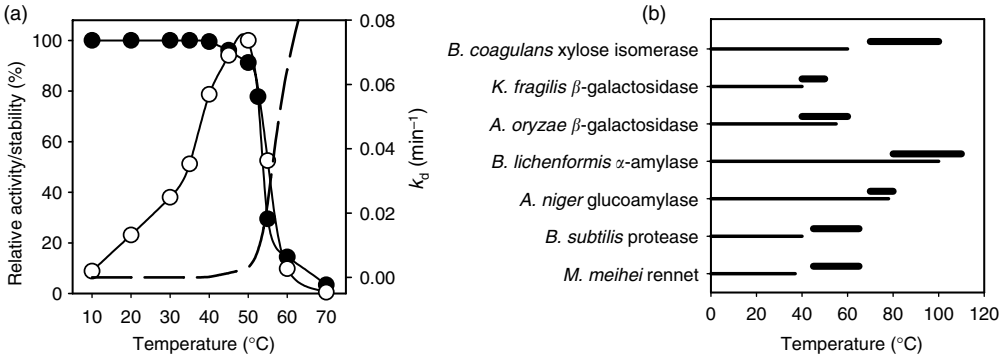


FIGURE 6.29 Thermal sensitivity of (a) pullulanase and (b) various commercial enzymes. (Data selected and figures redrawn from Godfrey, T. and West, S. (Eds.) (1996). *Industrial Enzymology*, 2nd edn., Stockton Press, New York and Ueda, S. and Ohba, R. (1972). *Agric. Biol. Chem.* 36:2382–2392.) Closed symbols represent enzyme stability, open symbols represent enzyme activity, and dashed line represents dependence of enzyme deactivation rate constant in panel (a). In panel (b), bold bars represent intrinsic optimum temperature range of the enzyme and upper range of the narrow bars indicate process temperatures where these enzyme are typically used.

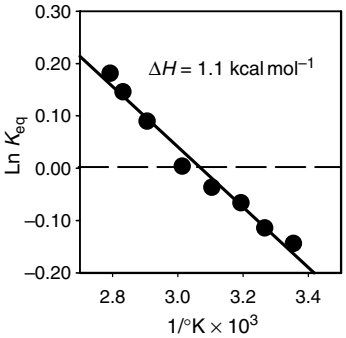


FIGURE 6.30 Thermal sensitivity of reaction equilibrium constant of xylose isomerase. (Figure redrawn from Rangarajan, M. and Hartley, B.S. (1992). *Biochem. J.* 283:223–233.)

to foods. The presence of solid phase domains, especially in phospholipid bilayers, constitutes a surface defect and creates access for lipolytic enzymes, often leading to enhanced hydrolysis.

6.4.1.3 Summary of Temperature Effects

While each enzyme exhibits unique behavior, some general observations can be made regarding enzyme thermal stability. Ligands (substrates or even inhibitors) improve stability by helping to retain native structure at and around the active site. Other compositional factors in the medium may also enhance or diminish thermal stability. Some general tendencies of enzyme thermal stability are that it is enhanced by decreasing size of the protein, lesser number of polypeptide chains, increasing number of disulfide linkages and salt bridges, elevated protein levels, being in a native over *in vitro* environment, for soluble over membranous proteins, and for extracellular over intracellular proteins.

6.4.2 pH Effects

6.4.2.1 General Considerations

All ionizable groups in proteins will undergo pH-dependent transitions based on intrinsic pK_a values of amino acid residues (Table 6.9 [41,113,140]). Many of these transitions will impact enzyme

TABLE 6.9
Ionization Properties of Amino Acid Ionizable Groups in Enzymes

Ionizable Group	pK_a (25°C)	ΔH_{ion} (kcal mol^{-1})	Ionizable Group	pK_a (25°C)	ΔH_{ion} (kcal mol^{-1})
Carboxyl			Ammonium		
C-Terminal (α)	3.0–3.2	$\sim 0 \pm 1.5$	N-Terminal (α)	7.5–8.5	10–13
β/γ-Carboxyl (ASP, GLU)	3.0–5.0		ε-Amino (LYS)	9.4–10.6	
Imidazolium (HIS)	5.5–7.0	6.9–7.5	Phenolic (TYR)	9.8–10.4	6.0–8.6
Sulfhydryl (CYS)	8.0–8.5	6.5–7.0	Guanidium (ARG)	11.6–12.6	12

Source: Fersht, A. (1985). *Enzyme Structure and Mechanism*, 2nd edn., W. H. Freeman & Company, New York; Segel, I.H. (1975). *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, Inc., New York; and Whitaker, J.R. (1994). *Principles of Enzymology for the Food Sciences*, 2nd edn., Marcel Dekker, New York.

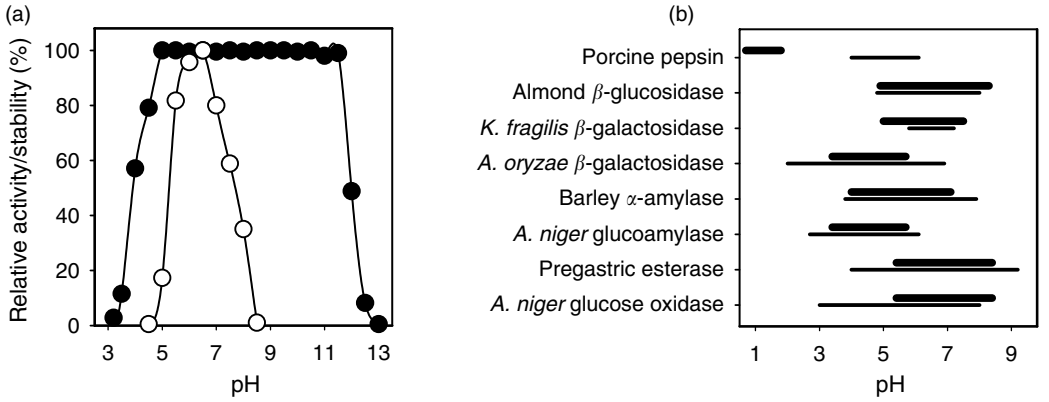


FIGURE 6.31 pH Sensitivity of (a) pullulanase and (b) various commercial enzymes. (Data selected and figures redrawn from Godfrey, T. and West, S. (Eds.) (1996). *Industrial Enzymology*, 2nd edn., Stockton Press, New York and Ueda, S. and Ohba, R. (1972). *Agric. Biol. Chem.* 36:2382–2392.) Closed symbols represent enzyme stability and open symbols represent enzyme activity in panel (a). In panel (b), bold bars represent where enzyme maintains >80% activity and narrow bars indicate where enzyme exhibits >80% stability.

stability, and over a narrow pH range they may act cooperatively to completely destabilize the enzyme (see Chapter 5). On the other hand, most amino acid side-chain ionizations have no or limited impact on enzyme *activity* and they remain "transparent" in the context of enzyme function. Rather, there are a limited number (often 1–5) of amino acid residues for which their ionization state confers pH-dependence of enzyme activity. Ionization of substrate, product, inhibitor, and cofactors may also have impact on enzyme reactivity, and pH may influence K_{eq} or equilibrium distribution of reactants in an enzyme reaction.

6.4.2.2 Enzyme Stability as a Function of pH

Enzymes have a characteristic dependence of stability on pH; an example is provided by the *A. aerogenes* pullulanase (Figure 6.31a [48,129]). Two general tendencies are worth mentioning (1) the pH range of enzyme stability is usually broader than the pH range for enzyme activity and (2) enzyme stability declines rapidly at destabilizing pH values, because pH destabilization is a cooperative process. In contrast, the decline in enzyme activity as a function of pH usually exhibits a

more measured transition with features of a titration curve, where 1–3 ionizable groups are the only determinants of the enzyme response to pH where each transition occurs. Enzyme stability to pH is measured by exposing (preincubating) the enzyme at various pH values, and then measuring residual activity at standardized conditions of (near-)optimum pH and a specific, nondenaturing temperature. A plot similar to that used to characterize k_d values for thermal sensitivity of enzymes can be used with pH replacing temperature as the variable of interest (Figure 6.28b). As with temperature sensitivity, enzyme stability to pH may be dependent on medium constituents and conditions; for example, the presence of substrate and other ligands may enhance pH stability, an example being the expansion of the pH range where α-amylase is >50% active from pH 4–7 to 4–11 in the presence of Ca^{2+} [140]. In some cases, pH-induced losses in activity may be reversible, but usually within a limited range of destabilizing pH values and for a limited duration. Pullulanase is inactive but stable at pH 9–11 for at least 30 min and within that period of time activity can be fully recovered by adjustment to pH 6–7 (Figure 6.31a).

Knowing pH stability of enzymes is obviously important for selecting an enzyme compatible with conditions prevailing for a potential application such that the enzyme will persist long enough to fulfill the expected function. It is also important to understand if enzyme destabilization contributes to a decline in activity at a given pH so that an analysis of pH effects on activity can be accurately interpreted (next section). Enzyme pH stability for selected commercial enzymes is shown in Figure 6.31b; pH stability ranges shown here are at temperatures encountered during processing, where stability is more limited than in the pullulanase example (where pH stability was measured at a nondenaturing temperature of 40°C). Likewise, temperature stability becomes reduced at pH ranges away from the optimum for stability of the enzyme. Thus, temperature and pH have coordinative influences on enzyme stability.

6.4.2.3 Effects of pH on Enzyme Activity [41,113,140]

Just like the catalytic locus of an enzyme comprises a few critical amino acids, the pH response of enzyme activity is also based on a few ionizable amino acids. The role of these amino acids can be (1) to confer conformational stability at the active site, or be involved in (2) substrate binding, or (3) substrate transformation, where the ionization state is critical to these roles. The pH range of >80% maximal activity at common processing temperatures for selected food enzymes also appears in Figure 6.31b.

To understand the basis for the effect of pH on enzyme activity, consider a typical "bell-shaped" pH dependence of activity often observed for enzymes (Figure 6.32a). The essential feature of this profile is the presence of separate alkaline- and acidic-side transitions, referred to as respective H^+-activating and H^+-deactivating steps. Thus, protonation of the alkaline pK_a group allows enzyme to function, and protonation of the acidic pK_a group attenuates enzyme function. Other types of pH

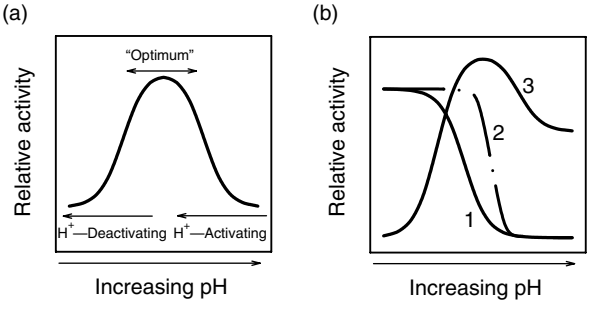


FIGURE 6.32 Typical responses of enzyme activity to pH. (a) A "bell-shaped" profile and (b) other types of pH behavior.

behavior shown (Figure 6.32b) include a single pH transition (plot 1), including one with a steeper decline in activity than the other (plot 2), and a case where a pH transition leads to a lesser active (instead of inactive) enzyme state (plot 3).

The empirical assessment of pH "optimum" of enzyme "activity" under specified conditions of enzyme assay (such as in Figure 6.31a) is rather arbitrary and has limited meaning. It is more informative to ascertain if the pH effect is on conformational stability, substrate binding, or substrate transformation. Thus, analysis of pH dependence of V_{max} and K_M provides insight into how enzyme function responds to pH. The pH behavior of critical enzyme ionizable groups is identical to other weak acids and bases:

$$EH \leftrightharpoons E^- + H^+ \quad \text{and} \quad K_a = \frac{[H^+][E]}{[EH]} \tag{6.32}$$

Such ionizations for the enzyme exist for both the "free" (E) and "bound" (ES) forms, and can be identified for each of the acidic- (K_{a1}) and alkaline-side (K_{a2}) transitions. Such behavior can be represented by three ionization states of the free enzyme:

$$HEH^+ \overset{K_{E1}}{\leftrightharpoons} EH + H^+ \overset{K_{E2}}{\leftrightharpoons} E^- + H^+ \tag{6.33}$$

where

$$K_{E1} = \frac{[H^+][HE]}{[HEH^+]} \quad \text{and} \quad K_{E2} = \frac{[H^+][E^-]}{[EH]} \tag{6.34}$$

The same pattern of behavior can be envisioned for the ES complex, where

$$HEH^+S \overset{K_{ES1}}{\leftrightharpoons} EHS + H^+ \overset{K_{ES2}}{\leftrightharpoons} E^-S + H^+ \tag{6.35}$$

where

$$K_{ES1} = \frac{[H^+][EHS]}{[HEH^+S]} \quad \text{and} \quad K_{ES2} = \frac{[H^+][E^-S]}{[EHS]} \tag{6.36}$$

Under this scenario, all ionization and kinetic equilibria can be assembled as just described in context with the catalytic steps of enzyme action (Figure 6.33 [28,113,140]). In this model, the most active enzyme states are the EH and EHS forms and they are associated with the optimum or "intrinsic" V_{max} and K_M values (consistent with Figure 6.32a). The model can be applied to determine if the decline in "activity" over the acidic or alkaline pH range is caused by certain enzyme forms (HEH^+ and E^-) not binding S, or those (HEH^+S and E^-S) incapable of transforming $S \rightarrow P$. The model also accommodates all enzyme species within a specified pH range being partially active (such as plot 3 in Figure 6.32b) with pH-modified kinetic constants ($\alpha/\beta K_M^{H^+}$ and $\alpha/\beta V_{max}^{H^+}$), with α/β modifiers typically in the range of $1 \rightarrow \infty$ and $1 \rightarrow 0$, respectively, for these kinetic constants. The terms $K_M^{H^+}$ and $V_{max}^{H^+}$ represent the dependence of these kinetic constants on pH relative to intrinsic K_M and V_{max} values at optimum pH.

With any enzyme, a reasonable assumption to make (based on the bell-shaped pH activity curve) is that three ionization states exist, and each has the potential to bind S with only the optimally ionized form capable of transforming $S \rightarrow P$. This assumption would modify the general model

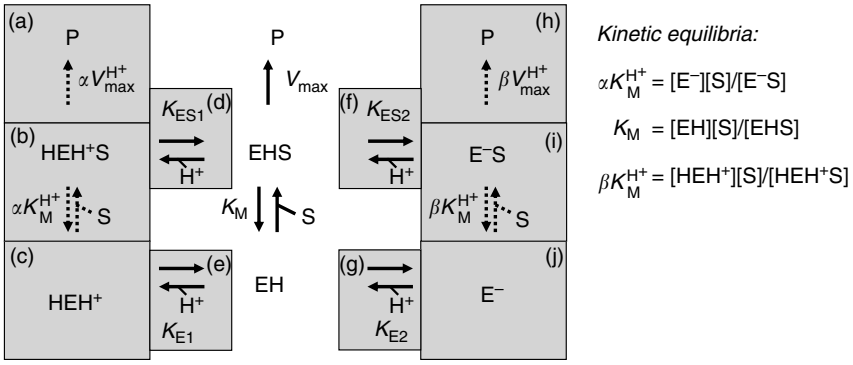


FIGURE 6.33 Kinetic model of enzyme activity response to pH. (Adapted from Copeland, R.A. (2000). *Enzymes. A Practical Introduction to Structure, Function, Mechanism, and Data Analysis*, 2nd edn., John Wiley & Sons, New York; Segel, I.H. (1975). *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, Inc., New York; and Whitaker, J.R. (1994). *Principles of Enzymology for the Food Sciences*, 2nd edn., Marcel Dekker, New York.)

(Figure 6.33) by omitting panels "a" and "h." If combined with the conventional reaction velocity equations applied earlier (Equation 6.15):

$$\frac{v}{E_T} = \frac{k_{cat} \times [EHS]}{[EH] + [HEH^+] + [E^-] + [EHS] + [HEH^+S] + [E^-S]} \tag{6.37}$$

$$\text{| ..... "E" species .....| |..... "ES" species .....|}$$

For the right side of the equation, all enzyme species can be expressed in the form of EHS, using the appropriate ionization (Equations 6.34 and 6.36) and kinetic (equations in Figure 6.33) equilibria. Since all enzyme species are in equilibrium, any particular enzyme species can be expressed in terms of any other enzyme species.

Next, factoring out EHS, factoring both sides of the equation by E_T (and using Equation 6.16), and then the numerator and denominator of the right-hand side by S/K_M, followed by K_M yields

$$v = \frac{V_{max} \times [S]}{K_M(1 + ([H^+]/K_{E1}) + (K_{E2}/[H^+])) + [S](1 + ([H^+]/K_{ES1}) + (K_{ES2}/[H^+]))} \tag{6.38}$$

$$\text{| ..... } f_E \text{ ..... | |..... } f_{ES} \text{ .....|}$$

This equation allows all free "E" species to be expressed collectively as a pH-dependent distribution term (f_E) called a Michaelis pH function, along with an analogous f_{ES} term for all "ES" species.* These functions reflect the quantitative distribution or ratios of the three ionization states of the "E" or "ES" species at any pH as a function of the H^+ and K_a terms (in essence, they yield "titration" curves). In addition, dividing numerator and denominator of the right side of Equation 6.38 by f_{ES} shows how key kinetic constants are influenced by pH:

$$v = \frac{V_{max}/f_{ES} \times [S]}{K_M((f_E)/(f_{ES})) + [S]} \tag{6.39}$$

* Note that these Michaelis pH functions were developed with the EHS species as the reference species; these functions can be developed for any "E" species as reference, and while they will take on different forms, enzyme behavior will be modeled identically for a given set of K_a and $[H^+]$ values.

and thus,

$$V_{max}^{H^+} = \frac{V_{max}}{(1 + ([H^+]/K_{ES1}) + (K_{ES2}/[H^+]))} \tag{6.40}$$

and

$$K_M^{H^+} = K_M \times \frac{f_E}{f_{ES}} = K_M \frac{(1 + ([H^+]/K_{E1}) + (K_{E2}/[H^+]))}{(1 + ([H^+]/K_{ES1}) + (K_{ES2}/[H^+]))} \tag{6.41}$$

If the ratio of these modified kinetic constants is taken:

$$\frac{V_{max}^{H^+}}{K_M^{H^+}} = \frac{V_{max}}{K_M \times (f_E)} = \frac{V_{max}}{K_M(1 + ([H^+]/K_{E1}) + (K_{E2}/[H^+]))} \tag{6.42}$$

Thus, the $V_{max}^{H^+}$ term relates *only* to the behavior of all "ES" species (f_{ES}), and the $V_{max}^{H^+}/K_M^{H^+}$ term relates *only* to the behavior of all free "E" species (f_E; also recall Equations 6.19 through 6.22) in how enzymes respond to pH.

Observations obtained for papain will help illustrate how pH affects enzyme function (Figure 6.34a–c [71]). A broad pH optimum of 5–7 is observed, and estimates of optimum V_{max} and K_M values allowed the data to be fitted (by the author) to the Equations 6.40 and 6.42 above for $V_{max}^{H^+}$ and $V_{max}^{H^+}/K_M^{H^+}$, yielding pK_a values of 4.0 and 8.2, and 4.2 and 8.2, respectively (Figure 6.34a,b). Values for pK_a can be identified from these plots by dropping perpendiculars from the points on the curves where the ordinate value represents 50% that of the maximum value observed. Since there

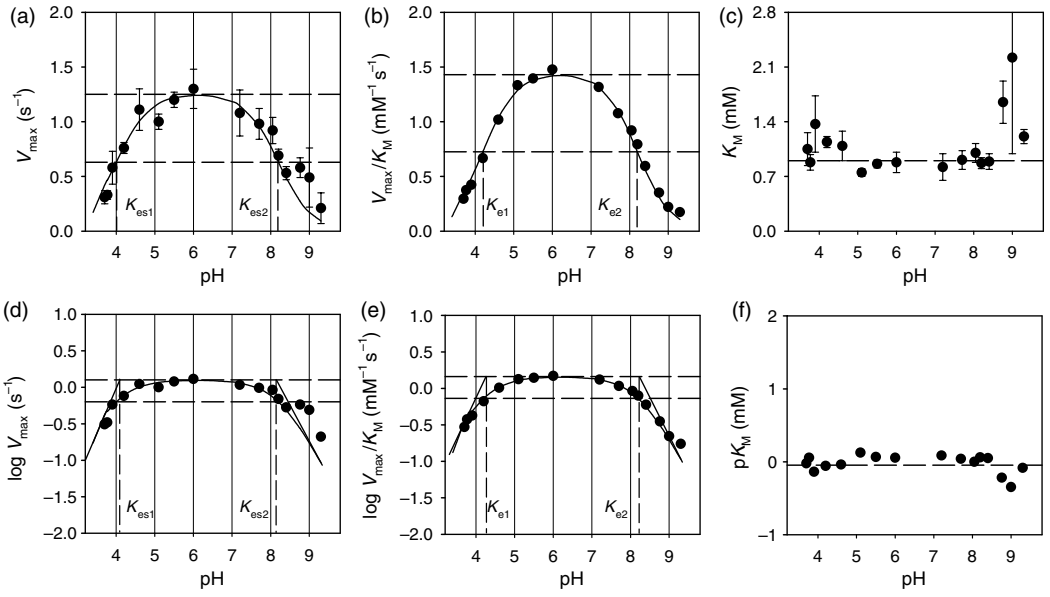


FIGURE 6.34 Analysis of enzyme activity response to pH using papain as an example. (Data obtained from Lowe, G. and Yuthavong, Y. (1971). *Biochem. J.* 124:117–122.)

was little change in K_M* as a function of pH (panel c), the pH-induced ionization of enzyme can be concluded to have negligible effect on substrate binding and can be solely attributed to a pH effect on the catalytic step over the pH region evaluated. To summarize for papain, a single ionizable group exists for each pH-transition, with all ionization states of the free E capable of binding S, but only the EHS form capable of transforming S → P. Thus, the model assumed leading to Equation 6.37 fits the behavior of papain, and panels a and h (Figure 6.33) would be omitted from the complete model with $\alpha = \beta = 1$ for $K_M^{H^+}$ to account for papain behavior. The response of papain activity (V_{max}) to pH in Figure 6.34a resembles that in Figure 6.32a.

To allow for more insightful analysis of pH effects [113], the log-transforms of Equations 6.40–6.42 yield

$$\log V_{max}^{H^+} = \log V_{max} - \log\left[1 + \frac{[H^+]}{K_{ES1}} + \frac{K_{ES2}}{[H^+]}\right] \tag{6.43}$$

$$\log \frac{V_{max}^{H^+}}{K_M^{H^+}} = \log \frac{V_{max}}{K_M} - \log\left[1 + \frac{[H^+]}{K_{E1}} + \frac{K_{E2}}{[H^+]}\right] \tag{6.44}$$

and

$$\log K_M^{H^+} = \log K_M - \log\left[1 + \frac{[H^+]}{K_{ES1}} + \frac{K_{ES2}}{[H^+]}\right] + \log\left[1 + \frac{[H^+]}{K_{E1}} + \frac{K_{E2}}{[H^+]}\right] \tag{6.45}$$

and observations are plotted routinely as "Dixon plots" (for papain behavior in Figure 6.34d–f). Equation 6.45 is not plotted per se, but $pK_M^{H^+}$ is instead ($p = -\log$), as this makes any downward deflection of the plot where a pH transition occurs correspond to impaired function, similar to the plots of Equations 6.43 and 6.44. The log forms of the equations make some aspects of enzyme behavior as a function of pH easier to visualize and interpret (Figure 6.34d–f). V_{max} is easily identified by the flat portion (slope ~0) of the plot, and the pH optimum is midpoint between the pK_a values. The slopes for the acidic ($+n$) and alkaline ($-n$) transitions at the most steeply ascending and descending portions of the pH response curve represents the number of ionizable amino acid residues involved in each transition. In the case of papain the Dixon plots yields slopes of +1 and −1, indicating that the ionization state of a single amino acid residue accounts for enzyme response to pH in each transition. Slopes of Dixon plots of enzyme function generally range 1–3, and multiple ionizable groups on the enzyme yield more cooperative transitions (such as plot 2 on Figure 6.32b).

Dixon plots also allow pK_a values to be estimated by two means. Since the point where pH = pK_a represents the condition where the ionizable group(s) is half-protonated, this corresponds to where the enzyme activity measured is 50% of the maximum. Thus, on a log scale used in Dixon plots, the pK_a values can be located where the pH response curve intersects a point 0.3 ordinate units below the maximum. Another way to estimate pK_a values is to extend the slopes of the ascending and descending portions to the intersection of the maximum response (a horizontal), and then drop perpendiculars to the axis to identify pK_a. Sometimes the choice of method used is dependent on the nature and extent of the data gathered. For papain (Figure 6.34d–f) estimates by both methods yields close agreement in pK_a values of 4.1 and 8.1, and 4.2 and 8.2, for the respective bound and free enzyme forms. Amino acid residues with ionizable groups that are consistent with these pK_a values are GLU/ASP and CYS (Table 6.9). However, the actual pH behavior of papain is conferred by an imidazole–thiolate (HIS–CYS) ion pair (which acts as a unit, see Figure 6.23). The CYS_{25} is active in the dissociated form while the HIS_{159} residue must be protonated for active site

* Changes in K_M of less than a few multiples are usually considered insignificant and must approach greater than or equal to threefold in magnitude of difference to be practically meaningful in pH response of enzyme action.

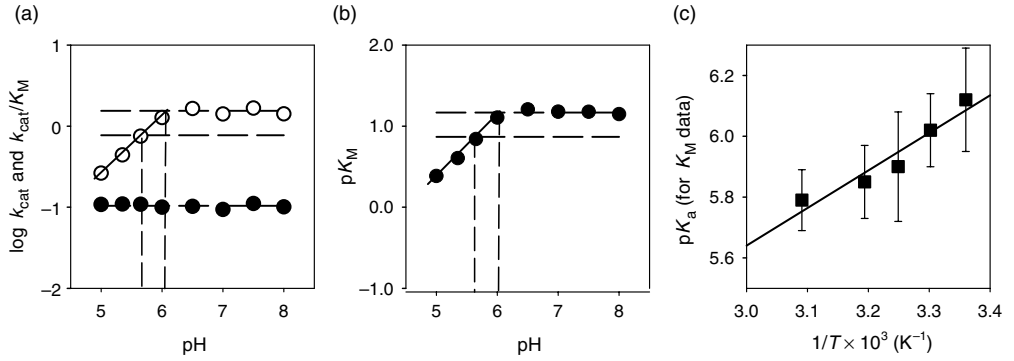


FIGURE 6.35 pH Response of xylose isomerase activity. Open circles represent k_{cat}/K_M and closed circles represent k_{cat} in panel (a). (Redrawn from Vangrysperre, W., et al. (1990). *Biochem. J.* 265:699–705.)

functioning. This behavior provides another example of how ionization properties of amino acids residues in proteins can be widely modulated relative to standard ionization potentials of amino acids in solution (Table 6.9).

With the preceding model, the pH-dependent behavior of enzymes can be quite broadly applied to any enzyme of interest. An analysis of the pH dependence of xylose isomerase indicates that over the pH range of 5–8 (commercial use is at pH 7–8), the ability of the enzyme to transform S → P is not affected (log k_{cat} curve is flat, Figure 6.35a [133]). However, the unit slope for the acidic transition indicates that the ionization state of a single ionizable group on the enzyme is responsible for substrate binding (K_M changes, Figure 6.35b), and since k_{cat} does not change, then $\Delta K_M \approx \Delta K_S$ for this analysis. The purpose of identifying pK_a values that represent critical pH-sensitive transitions in enzyme functioning is to insinuate the identity of the amino acid residues involved in that enzyme response. The pK_a value of the ionizable group in xylose isomerase is 5.7–6.1, making it likely to be an HIS residue (Table 6.9). The van't Hoff relationship (Figure 6.30a, Equation 6.31) is often used to further insinuate the participating amino acid residues based on characteristic ΔH_{ion} values. For xylose isomerase, the pK_a of the ionizable group changed as a function of temperature with a ΔH_{ion} value of 5.6 kcal mol^{-1}(from slope, Figure 6.35c), also consistent with that observed for imidazole residues (Table 6.9).

6.4.2.4 Other Types of pH Behavior

Other types of pH behavior can affect enzyme reactions. Ionization state of substrate, product, or inhibitor may influence enzyme reactivity depending on the nature of the interactions that allow enzyme to bind and transform these ligands. Likewise, ionization of enzyme amino acid side chains may modulate selectivity of reaction among potential substrates. For example, many proteases exhibit different pH optima for hydrolytic activity toward different protein substrates [48].

6.4.3 Water Relations and Enzyme Activity [36,39,112]

Control of the level and disposition of water in foods is a principal form of preservation and can affect enzyme activity and stability. Water impacts rates of reactions by serving as a diffusion medium, controlling dilution or concentrations of solutes, stabilizing and plasticizing proteins, and serving as cosubstrate for hydrolytic reactions. Reducing the amount of bulk or solvent water (by dehydration or freezing) evokes several interrelated compositional and material changes in foods that influence enzyme reactions.

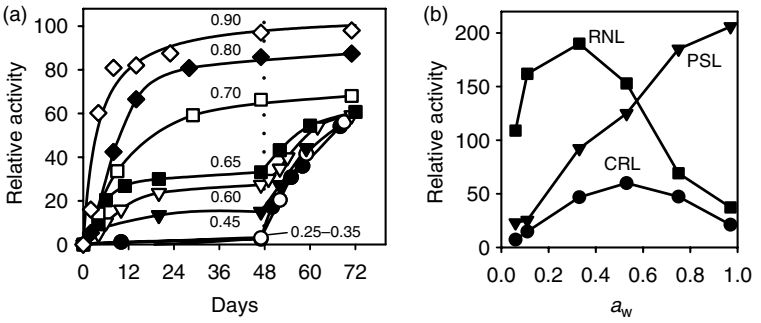


FIGURE 6.36 Response of enzyme activity to a_w. (a) Response of ground barley malt (source of phospholipase) on 2% lecithin at 30°C, with adjustment to a_w of 0.70 after 48 days for initial a_w of 0.25–0.65. (b) Response of ester synthesis activity of various lipases (RNL = *Rhizopus niveus* lipase; PSL = *Pseudomonas* spp. lipase; CRL = *Candida rugosa* lipase). (Redrawn from Acker, L. and Kaiser, H. (1959). *Lebensm. Unters. Forsch.* 110:349–356 [in German] and Wehtje, E. and Adlercreutz, P. (1997). *Biotechnol. Lett.* 11:537–540.)

6.4.3.1 Desiccation and Water Activity Effects

The principal effects of reducing bulk or solvent water is to diminish the role of water in acting as a diffusion medium and as cosubstrate. The extent of water reduction is best characterized by the thermodynamic term of water activity (a_w), as it relates to how water behaves with respect to solutes (including enzymes). For lysozyme as an example, at a_w 0 to 0.1, water is tightly bound (monolayer) to charged and highly polar groups on proteins. At a_w 0.1–0.4, water becomes bound to less polar domains of protein including the peptide backbone. At $a_w > 0.40$ water of condensation contributes to multilayer water and increasingly to the fraction of true bulk or solvent water. The exact a_w values where similar transitions in states of water occur in food matrices are material dependent. The effect of a_w on enzyme reactions was addressed in the 1950–1980s, and a generally applicable example of behavior is illustrated in Figure 6.36a [1,139]. As a_w is reduced within the range of 0.90 to 0.35, the progress of hydrolysis reactions is slowed, and approaches a near-equilibrium position of more limited extent of hydrolysis. When a_w is then elevated, reaction progress resumes in a manner that is representative to that initially occurring at that a_w. Thus, this effect of water is reversible, and in food and biological matrices such behavior is interpreted as capillary effects that limit the extent of reaction progress at a limiting a_w. Such effects have been shown for lipase, phospholipase, and invertase activity, but they are generally applicable to all enzymes, as polyphenol oxidase activity is reduced by 90–95% in terms of initial rate and extent of reaction as a_w is reduced from 1.0 to 0.60 [126]. For ester synthesis reactions, lipases from various sources have different and distinct a_w optima (Figure 6.36b).

Enzymes exhibit different minimum a_w for catalytic function. At a_w at or below the monolayer, enzyme plasticity is limited, but some enzymes still exhibit activity. Less than monolayer water may restrict reactivity, but this also enhances thermal stability, since conformational freedom is also restricted and there is less tendency for protein unfolding at otherwise denaturing temperatures. The threshold or minimum a_w required for enzyme activity ranges 0.25–0.70 for several oxidoreductases, and 0.025–0.96 for several hydrolases, in both food matrices and model systems (Table 6.10 [36]). Even low residual enzyme activity may be sufficient to have impact on food quality given the long times that intermediate moisture foods are stored.

Another effect of reducing a_w is to influence equilibria involving water (hydrolysis reactions) through mass action effects. Thus, for $AB + H_2O \leftrightarrows A' + B'$

$$K_{eq} = \frac{[A'] \times [B']}{[AB] \times [H_2O]} \tag{6.46}$$

TABLE 6.10
a_w Requirements for Activity of Selected Enzymes

Enzyme	Matrix/Substrate	Minimum a_w	Enzyme	Matrix/Substrate	Minimum a_w
Amylases	Rye flour	0.75	Amylases	Starch	0.40–0.76
	Bread	0.36			
Phospholipases	Pasta	0.45	Phospholipases	Lecithin	0.45
Proteases	Wheat flour	0.96	Lipases	Oil, tributyrin	0.025
Phytase	Grains	0.90	Phenol oxidase	Catechol	0.25
Glucose oxidase	Glucose	0.40	Lipoxygenase	Linoleic acid	0.50–0.70

Source: Drapon, R. (1986). In *Food Packaging and Preservation. Theory and Practice*, Mathlouthi, M. (Ed.), Elsevier Applied Science Publishers, New York, pp. 181–198.

As a_w decreases, there is a shift in position of reactants and products toward accumulation of [AB]. This principle is exploited commercially by using lipases in microaqueous media ($<1\%$ H_2O) to cause various reactions (Figure 6.26) leading to the production of lipids with improved functionality. Similarly, the optimum water content (related to a_w) is ~2–3% for thermolysin reactions leading to peptide bond formation in the course of Aspartame synthesis [78]. Many enzymes exhibit an optimum a_w for activity and it is usually >0.90.

The combined lack of diffusion medium and enzyme plasticity may cause changes in reaction pathways and product distribution [36,112]. For α-amylase action on starch, as a_w is reduced from 0.95 to 0.75, there is a shift in maltooligosaccharide product distribution from a heterogeneous mixture of oligomers of 1–7 glucose units to that favoring products of 1–3 glucose units. This indicates that hydrolysis is no longer random in nature. Restricted diffusion of enzyme and substrate favors greater processivity in enzyme attack, since the limited mobility of reactants may subject starch segments to multiple and proximal hydrolytic actions. Similarly, restricted diffusibility at a_w of 0.65 renders lipoxygenase reaction end-products elevated in linoleate condensation products with a corresponding diminution in fatty acid hydroperoxides. The limited ability for diffusion allows the hydroperoxides to remain in proximity longer and participate in bimolecular free radical addition (condensation) reactions.

Reduction in a_w may also change kinetic or equilibrium constants governing enzyme reactivity. For example, the pH optimum of polyphenol oxidase shifts >0.5 pH unit as a_w is decreased from 1.0 to 0.85 [126]. Such a change is consistent with diminished dielectric character of the medium and a corresponding increase in pK_a of important ionizable groups important to enzyme function. Lipase exhibits a minimum K_M at a_w of ~0.4 [36], and this may result from a change in properties of the enzyme or nature of the substrate interface.

Depending on the composition and a_w of some food or model system matrices, glass transitions may occur, where molecular motion is greatly restricted relative to a "rubbery" or more fluid state (Chapter 2). In some cases the glassy state is more stabilizing to enzymes, but enzymes generally exhibit a temperature-dependent sensitivity of stability in low moisture media, regardless of whether a glassy or rubbery state exists [108]. In terms of enzyme activity, studies on model systems have indicated no obvious elevation of enzyme activity as may be expected when a transition from the glassy to rubbery state occurs [24]. Specific compositional factors may modulate enzyme activity and/or stability in low moisture systems more so than the mere presence of a glassy state.

Finally, as water is removed there is a corresponding decline in viscosity of the remaining liquid phase, and this may serve to attenuate enzyme reactions by reducing diffusibility of reactants and products. The effect of viscosity has been evaluated in a few cases of enzyme action, using inert "viscogens" (e.g., glycerol, polyols, polymers). Increases in viscosity have been shown to reduce enzyme reaction rates that are diffusion controlled or when they cause a change in rate-limiting

step, such as to the product dissociation step. Diffusion-controlled (near-perfect) enzyme reactions are considered to be those with k_{cat}/K_M values of $\sim 10^8$–10^9 $M^{-1}s^{-1}$, approximating diffusion-limited rates for bimolecular reactions between a large and small molecule [138]. Early findings of attenuation of invertase reactions at high [sucrose] were interpreted as an effect of increased viscosity, but it was later shown to be caused largely by substrate inhibition [76]. This example emphasizes the difficulty in trying to isolate the individual effect of an environmental factor as water content is modified, since many other factors are simultaneously modified.

6.4.3.2 Osmotic Effects of Desiccation [39,148]

As water is progressively removed from foods, or as solutes are added to a liquid medium, dissolved solutes become more concentrated in the remaining liquid aqueous phase. Consequently, another outcome of desiccation is increased ionic strength and osmolality. Stability, and to a large extent activity of enzymes in hyperosmotic media, is influenced by the profile and concentrations of solutes present; specific ionic constituents are generally classified as salting-in (destabilizing) or salting-out (stabilizing) toward proteins (Chapter 5). Each enzyme exhibits a characteristic response to these solutes and changes in their concentrations as desiccation takes place. Relevant to enzyme behavior in hyperosmotic media are many commercial enzyme processes making use of high levels (10–40%) of substrate (pectinases, proteases, amylases, and sugar-transforming enzymes). Fortunately, many of these substrates are also protein-stabilizing agents, such as polyols, sugars, and amino acids [148], and high substrate levels help stabilize enzymes to thermal denaturation.

Another consequence of enzyme reactions in high solids media is the favoring of reverse reactions (especially hydrolyses) by mass action effects (recall Equation 6.46). Reverse reactions with lipases provide the means for synthesizing or rearranging esters (Figure 6.26). Plasteins formed by proteases at elevated [peptide] are mediated by transpeptidation reactions. Such reactions allow the incorporation of nutritionally limiting amino acids. Use of glucoamylase under commercially relevant conditions (Figure 6.19) yields a limited level of undesirable isomaltose (α,1–6 linkage) accumulation through reverse hydrolysis reactions. β-Galactosidase mediates glycosyltransfer reactions at high [lactose] and yield oligomers of galactose and glucose that have potential use as prebiotics.

Some enzymes are constantly exposed to hyperosmotic stress in nature. Examples of organisms living in hyperosmotic environments include all marine species (salt water is $\sim$3.5% NaCl), and plants and microorganisms inhabiting brackish water, high salinity soils, mineral springs, and deep-sea vents. Freezing and desiccation also brings about hyperosmotic conditions. Osmoregulatory systems have evolved to mitigate the negative effects of high osmotic and ionic strength media. Osmoprotectants are compounds such as polyols (glycerol, mannitol, sorbitol), sugars (sucrose, glucose, fructose, trehalose), amino acids (especially GLY, PRO, GLU, ALA, β-ALA), and a series of methylated amines (Figure 6.37). Among these structures, note the frequency of the stabilizing functional groups of $-$OH (H-bonding capability), NH_4^+, $R_xNH_y^+$, $-CH_2-COO^-$, and SO_3^{2-}. Such groups stabilize proteins by countering or minimizing the effect of destabilizing agents such as Na^+, K^+, Cl^-, urea, and ARG.

The mechanisms by which these osmoprotectants act are believed to include steric repulsion between solute–protein (promoting water structure and protein compactness, promoting the native state) and direct solute–protein interactions (H-bonding). Two examples of osmoprotection deserve special mention, those by methylated amines and trehalose. Tissues of marine organisms can be comprised of up to 100 mM trimethylamine-*N*-oxide (TMAO). This endogenous osmolyte protects tissue enzymes from destabilizing effects (adverse changes in K_M) of salt and even urea (a potent protein denaturant existing in tissues of sharks and rays). A related compound, betaine, relieves inhibitory effects of NaCl on enzymes in saline-stressed plant tissues. Trehalose (glucopyranosyl-α-1,1-glucopyranoside) is among the most effective osmoprotectants known. It appears to H-bond with protein and also promotes structure of water in stabilizing proteins to desiccation and freezing stress [148]. While osmoprotectants preserve enzyme activity in host tissues in water-stressed

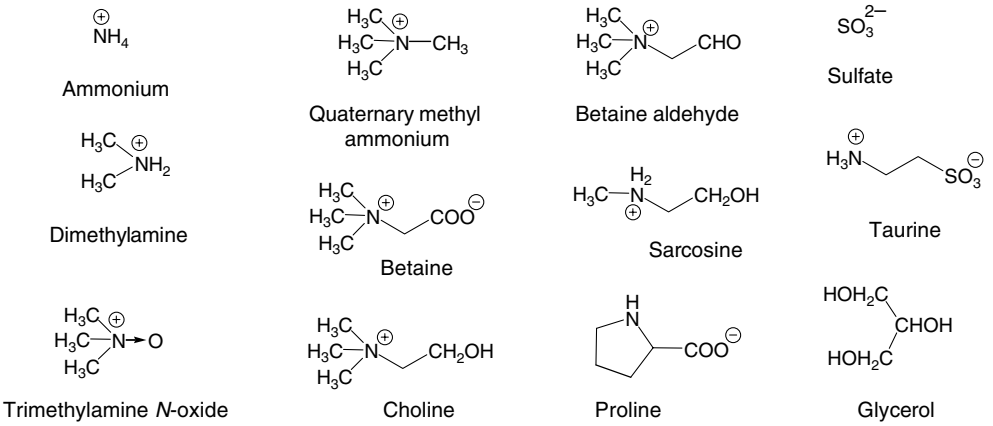


FIGURE 6.37 Osmolyte systems.

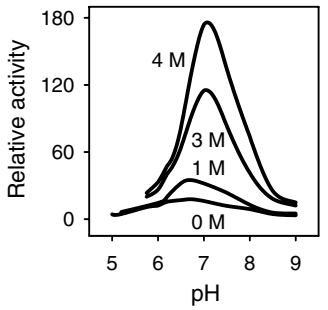


FIGURE 6.38 Salt (NaCl) activation of thermolysin activity. (Redrawn from Inouye, K., et al. (1997). *J. Biochem.* 122:358–364.)

environments, they can also be added to enzyme preparations to render them more stable. Such is done for freezing and freeze-drying of enzyme preparations, many of which are $\leq$10% active protein with the rest being excipient or carrier material that may include cryo-/osmoprotectants.

Some enzymes may require ionic constituents to function optimally or they have evolved to function well under conditions of water stress, such as those from halotolerant or halophilic organisms. Some of these enzymes have been identified empirically through the evolution and use of various starter cultures for fermentations where added salt is involved (e.g., cheese, soy sauce). Enzymes of significance to fermentations must be tolerant enough to persist and be sufficiently active to cause the desired change during a proper fermentation. In other cases, salt (osmotic) stimulation of activity has been observed. Thermolysin, used to synthesize the sugar substitute Aspartame, is stimulated 12-fold by 4 M NaCl at optimum pH ~7 (Figure 6.38 [58]), and stimulation by monovalent cations occurs in descending order: $Na^+ > K^+ > Li^+$ [58]. Stimulation affects only the k_{cat} step and not S binding, and shifts the acidic group pK_a from 5.4 to 6.7, while the alkaline group remains at pK_a of ~7.8. The high salt environment activates the enzyme through electrostatic interactions at the enzyme surface and active site, and this is associated with a conformational change in the protein. This enzyme is ideally suited for peptide synthesis at high cosubstrate levels.

6.4.3.3 Desiccation by Freezing

Freezing is distinct from other desiccation processes on account that bulk water is removed as a solid phase and this is accompanied by lower temperatures (<0°C) than encountered in dried, intermediate moisture, or high osmotic foods. Thus, temperature and increased solute concentration

are the principal determinants of enzyme activity in frozen media, with viscosity and diffusion effects embedded in both of these factors. a_w may be a less important factor in frozen compared to desiccated media because it is dictated by the relative vapor pressure of ice and supercooled water at the same temperature; a_w is only suppressed to 0.82 at –20°C (Chapter 2). Freezing effects were examined closely in the 1960–1980s, although efforts continue because of the sustained interest in cryopreservation of biological systems. Studies have made use of model systems as well as reactions in food matrices.

The combined influence of the two dominant factors accounting for freezing effects on enzyme reactions can be summarized as follows. Reduced temperature will always reduce k_{cat} and predict a declining rate of reaction based on the characteristic E_a for the reaction. Concentration effects are more varied and relate to the initial concentration of agents controlling enzyme activity (substrates, inhibitors, affectors, cofactors, buffering agents) in the unfrozen medium and the collective effects of elevating their concentrations by removal of solvent water as ice. Concentration of solutes may have negative impact or destabilize enzymes through osmotic and/or inhibitor effects, especially if $S > K_M$, and reaction rates will decline as the outcome. The net result would be an overall decrease in reaction rate upon freezing. A limited enhancement of enzyme reactivity, such as through the elevated concentration of S or positive affector, would be expected to enhance reactivity, but in a manner that may roughly balance the effect of temperature, resulting in little or no change upon freezing. The third potential outcome is where the substrate-concentration effect substantially enhances reactivity, especially for initially dilute [S], such that this effect is dominant over temperature and there is a net increase in reactivity upon freezing.

The physical event of ice crystal formation can have at least three distinct consequences. One is that in cellular systems, ice crystals can disrupt cellular structures and promote mixing of enzyme and solutes that may originate in different cellular compartments. This decompartmentation effect often is responsible for cellular systems exhibiting enhanced reactivity at high, abusive freezing temperatures (−3 to −12°C), and sometimes as low as −20°C. Ice crystal size, which is primarily a function of how fast freezing occurs (and secondarily through the process of recrystallization), can also have effects on enzyme reactivity in frozen systems. Fast freezing will favor greater homogeneity in ice crystal distribution and smaller, more dispersed "pools" of the remaining reactive, liquid phase. This may retain some segregation between enzyme and reactants, especially if they were originally contained in different cellular compartments, even though the net concentration effect of freezing would be equivalent to slow freezing to the same end-point temperature. The third consequence relates to freezing rate, and while it has long been considered that the faster the freezing in the range of ∼1–100°C min^{-1}, the better enzyme activity/stability is retained, the opposite seems to be more the general rule [19,122]. Faster freezing creates smaller ice crystals with greater surface area than slow freezing, and with less opportunity for pooling of unfrozen liquid media. The small crystals appear to foster surface denaturation of enzymes. Some proteins are not as sensitive as others, and in cellular systems, cellular barriers and compartments may mitigate or exacerbate this phenomenon. In any event, slow to moderate rates of freezing favor stability and retention of enzyme activity during frozen storage. Generally, enzyme activity is lost during sustained frozen storage of aqueous systems, but this occurs to a more limited extent in lyophilized powders, where ice is removed before storage.

Thawing rates have profound influence on retention of enzyme activity in biological media. Progressively slower thawing rates, from 10°C min^{-1} to 0.1°C min^{-1}, lead to increasing losses of several enzymes in model solutions, and the temperature range where most deactivation occurs is from −10°C to thawing [19,42]. Ice recrystallization during thawing may cause additional surface tension and shear to further denature proteins during this process. Slow thawing was also observed to be particularly denaturing to enzymes in food matrices, with onion alliinase serving as an example [136].

Increased viscosity in the liquid phase is another consequence of freezing, with less water available to serve as a diffusion medium. As was observed for reductions in a_w, lower freezing temperatures limits the rate and extent to which reaction can occur (Figure 6.39 [10,40]). More

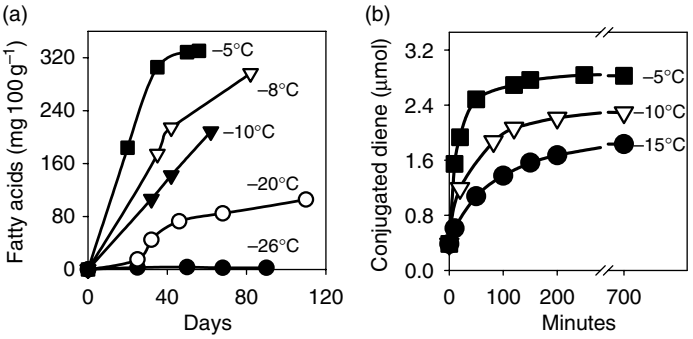


FIGURE 6.39 Effect of freezing on reaction progress of (a) lipase action in unblanched peas and (b) lipoxygenase oxidation of linoleic acid in model reactions. (Redrawn from Bengtsson, B. and Bosund, I. (1966). *J. Food Sci.* 31:474–481 and Fennema, O. and Sung, J.C. (1980). *Cryobiology* 17:500–507.)

recently, an attempt was made to quantify the effect of viscosity by the study of alkaline phosphatase in frozen sucrose solutions [23]. Alkaline phosphatase is widespread in nature and in milk it is used as a thermal process indicator; it is an efficient enzyme that reacts at a rate (k_{cat}/K_M of 10^6–10^7 $M^{-1}s^{-1}$) near the limit of diffusion. Measurements of catalytic function (k_{cat}/K_M) were in good agreement with the predicted effect of viscosity and could account for behavior in partially frozen solutions. However, other factors may be important for enzymes that react at less than diffusion-controlled rates. One of these other factors not yet discussed include eutectics that can cause ionic and compositional (pH) changes that can impact enzyme activity and stability. Also impacting enzyme sensitivity to freezing are enzyme concentration and protein concentration in the medium, with greater concentrations favoring greater degree of retention of active enzyme, likely through stabilizing protein–protein interactions. Last, the presence of cryoprotectant compounds improves enzyme stability, with the same osmoprotectants as discussed earlier being important, particularly trehalose, other polyols, and sugars.

6.5 ENZYMES ENDOGENOUS TO FOODS AND THEIR CONTROL

The balance of this chapter deals with the characterization and manipulation of enzyme activity endogenous to foods, a continuing challenge to food scientists. The intent here is to provide an understanding of the nature and disposition of enzymes in tissues, the complexity of their behavior and interactions, and how physical and chemical strategies may be employed to attenuate or potentiate enzyme action where necessary or desirable. Complex and interrelated biochemical events, such as ripening and postharvest and postslaughter metabolism, as well as genetic manipulation, will not be covered as they are presented in other chapters.

6.5.1 Cellular and Tissue Effects

Enzymes related to food quality or processing are often studied in purified or partially purified forms to provide an understanding of the intrinsic properties and characteristics of the enzyme. Such *in vitro* studies often make use of enzyme levels of 10^{-7}–10^{-12} M. A quick calculation using a hypothetical food that is 10% protein of 1000 different proteins with an average mass of 100 kDa, yields an estimate of the average concentration of any protein species as being 10^{-6} M [113]. Of course some individual proteins are more enriched than others, so the range of concentrations can easily be ±3 orders of magnitude (10^{-3}–10^{-9} M). Thus, on average the levels of enzymes in foods and biological matrices are several orders of magnitude greater than used in studies to characterize

TABLE 6.11
Examples of High Concentrations of Enzymes in Foods and Tissues

Enzyme	Source	Level Found	Concentration (mM)	Comment
Glyceraldehyde-3-phosphate dehydrogenase	Muscle (meat)	>1% weight, wet basis	0.34	Related metabolic enzymes: aldolase is 0.15 mM; lactate dehydrogenase is 0.11 mM; multienzyme complexes exist
Peroxidase	Horseradish root	20% of protein	0.2	Isoforms may be cytosolic or plastidic
Lipid acyl hydrolase	Potato tubers	~30% of protein	0.2	Storage protein, localized at extra-vacualor membrane, enriched at bud end of tuber
Alliinase	Onion bulb	~6% of protein	0.02	Vacuolar (onion), or enriched in bundle sheaths (garlic)
	Garlic clove	~10% of protein	0.2	
Panceatin (mixture of digestive proteases)	pancreas	~0.04 g/g dry weight	~1.0 total protease	Trypsin, chymotrypsin, and elastase may exist as zymogens and active forms

them. Examples of high enzyme levels in foods from various sources are provided in Table 6.11. The levels estimated in this table do not account for any further enrichment conferred by localization (compartmentation) within the cell that can increase concentrations by another order of magnitude or more. Even nontissue foods such as milk and eggs exhibit structural heterogeneity that serves to distribute and concentrate endogenous components among discrete phases.

Compartmentation and *in vivo* concentration of enzymes impact their properties in foods in several ways. Properties of enzymes can be dependent on concentration. This is especially true for oligomeric enzymes where dissociation is favored upon dilution, and thus kinetic character associated with oligomeric enzymes (allosterism) may be diminished. Kinetic relationships between E and S may also change with changes in [E], even though theoretically, constants such as K_M are independent of [E]. A striking example is available for muscle phosphofructokinase (which influences the rate of postmortem glycolysis during the conversion of muscle into meat) (Figure 6.40a [9,83]). At physiologically relevant levels of enzyme (500 $\mu g\ mL^{-1}$, $\sim 10^{-6}$ M), $S_{0.5}$ is 0.5 mM, whereas at 5 $\mu g\ mL^{-1}$ ($\sim 10^{-8}$ M), $S_{0.5}$ is approximately tenfold greater at 6.4 mM. Furthermore, at the lower level of enzyme, inhibition by ATP (also a cosubstrate) in the presence of activator, fructose-2,6-biphosphate, was more acute with a K_I of 1.2 mM, compared to a K_I of 10 mM at physiological levels of enzyme. Another dimension of enzyme behavior *in situ* is the fact that other constituents can modulate reactivity. In the presence of fructosebisphosphatase, phosphofructokinase exhibits hyperbolic-type kinetics with a $S_{0.5}$ of 2.9 mM, whereas alone, it exhibits allosteric kinetics with an increased $S_{0.5}$ of 9.2 mM (Figure 6.40b). Thus, fructose bisphosphatase may "activate" phosphofructokinase in muscle *in situ* through structural or metabolic effects.

Another factor impacting enzyme reactivity *in situ* is the relative levels of enzymes and substrates and cofactors, the latter two for which multiple enzymes may compete. For example, intermediate metabolites of glycolysis range 20–540 μM, whereas glycolytic enzymes range 32–1400 μM [121]. Thus, substrates may be limiting to reactions for both primary and secondary metabolic pathways. Steady-state levels of NAD^+/NADH are estimated to be ~ 540/50 μM, and competition and relative K_M values for these cosubstrates among the many oxidoreductases in biological systems often dictates which enzymes are active and which are not (there is virtually no "free" NAD^+/NADH). In contrast, *in vitro* characterization of enzyme activity often makes use of excess (co)substrate(s) and [S] of 10^{-6}–10^{-2} M.

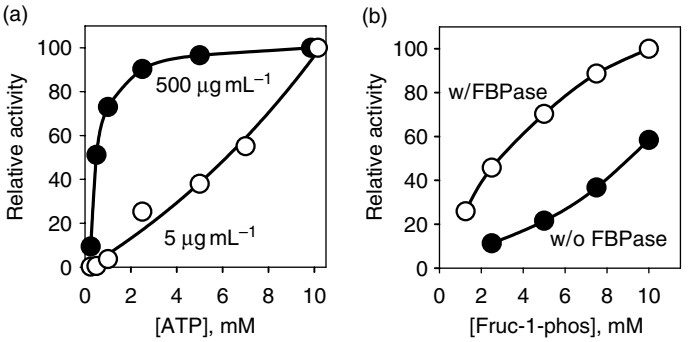


FIGURE 6.40 Effect of simulated *in situ* conditions on functioning of (a) phosphofructokinase and (b) phosphofructokinase in the presence or absence of fructosebisphosphatase (FBPase). (Redrawn from Bär, J., et al. (1990). *Biochem. Biophys. Res. Comm.* 167:1214–1220 and Ovádi, J., et al. (1986). *Biochem. Biophys. Res. Comm.* 135:852–856.)

It should be evident by now that compartmentation is a key feature of controlling enzyme action in foods and biological systems. However, compartmentation means more than simply a separation by a membrane structure, within an organelle or some other physical barrier. Enzymes can be separated from other enzymes or their substrates by being bound to other proteins, membranes, or even polysaccharides. Enzymes can be cocompartmented by interacting and binding to each other and this association allows for metabolic channeling of substrates and intermediates to end-products by segregating them from the cytosolic or diffusional metabolic pool in cells. Enzymes may also be functionally compartmented as latent forms by other factors. Examples include localized pH or ionic strength (or gradients), presence of a reversible inhibitor, lack of positive affector or cofactor, or requirement of proteolytic activation of zymogen forms of enzymes.

The disposition of enzymes in foods may be quite easily controlled in some cases. The simple act of disrupting tissue is one means. Whether this improves quality (as in flavor generation), or detracts from it (enzymic browning), depends on the specific food material, its specific quality attributes, and the particular reaction evoked. For example, lipoxygenase action on lipids may yield either rancid or pleasant flavors, and enzymic browning is desirable in tea chemical "fermentation" but not for fresh-cut fruit and vegetables.

6.5.2 Enzyme Activities Related to Color Quality of Foods

6.5.2.1 Phenol Oxidases [119,130,137]

Enzymic browning is caused by enzymes collectively referred to as phenolase, phenoloxidase, polyphenol oxidase, catecholase, cresolase, and tyrosinase. These enzymes are widespread in microorganisms, plants, and animals, including humans where its action leads to skin pigmentation. These enzymes are related by having the same Type-3 (oxidatively coupled) binuclear copper active site architecture and can mediate the latter or both of the following reactions:

$$\text{monophenol} + O_2 + 2H^+ \rightarrow o\text{-diphenol} + H_2O \tag{6.47}$$

$$o\text{-diphenol} + \tfrac{1}{2}O_2 \rightarrow o\text{-quinone} + H_2O \tag{6.48}$$

The first reaction is hydroxylation and is classified as monophenol monooxygenase (EC 1.14.18.1) activity, while the second reaction is oxidation and is classified as 1,2-benzenediol:oxygen oxidoreductase (EC 1.10.3.1) activity. The former reaction provides the basis for "cresolase activity," since *p*-cresol generally represents monophenols and it is routinely used as a substrate for monophenol

hydroxylation (and subsequent oxidation). Catechol is the common name for 1,2-benzenediol (the simplest *o*-diphenol), and thus, cresolase and "catecholase" activities are used to represent the respective hydroxylation and diphenol oxidation steps. Tyrosinase is a term used to generally represent enzymes with both hydroxylation and oxidation reactions, and the name derives from the enzyme abundant in common mushroom (*Agaricus bisporus*), which acts on the endogenous substrate tyrosine. Enzyme action does not form brown pigments directly. Rather, the *o*-quinones resulting from enzyme action undergo chemical condensation reactions (may involve amines and proteins) to yield diverse, polymeric, and conjugated products called "melanins" that are collectively reddish-brown in color.

Each atom of binuclear copper is tightly liganded to three HIS residues (sweet potato catecholase as reference; $HIS_{88,109,118}$ and $HIS_{240,244,274}$), and this feature is the most highly conserved sequence among phenol oxidases and related binuclear copper enzymes [38,119]. Higher plant enzymes tend to be monomeric or homo-oligomers of 30–45 kDa monomeric mass. Tyrosinases are often glycosylated and exist in multiple isoforms exhibiting different substrate selectivities. The mechanism for tyrosinases involves redox reactions in $2e^-$ steps (Figure 6.41 [38,119]). The state of the enzyme in tissues is distributed as ~85% MET (Cu^{II}–Cu^{II}–OH^-) and ~10–15% OXY (Cu^{II}–Cu^{II}–O_2^{2-}) forms, and the enzyme is often isolated as the MET form. Oxidation of diphenols is facile with either form, and reactions proceed quickly through the cycle shown on the perimeter. Thus, in one complete cycle, one mole O_2 and $4e^-$ from substrate are used for two moles H_2O produced. In the

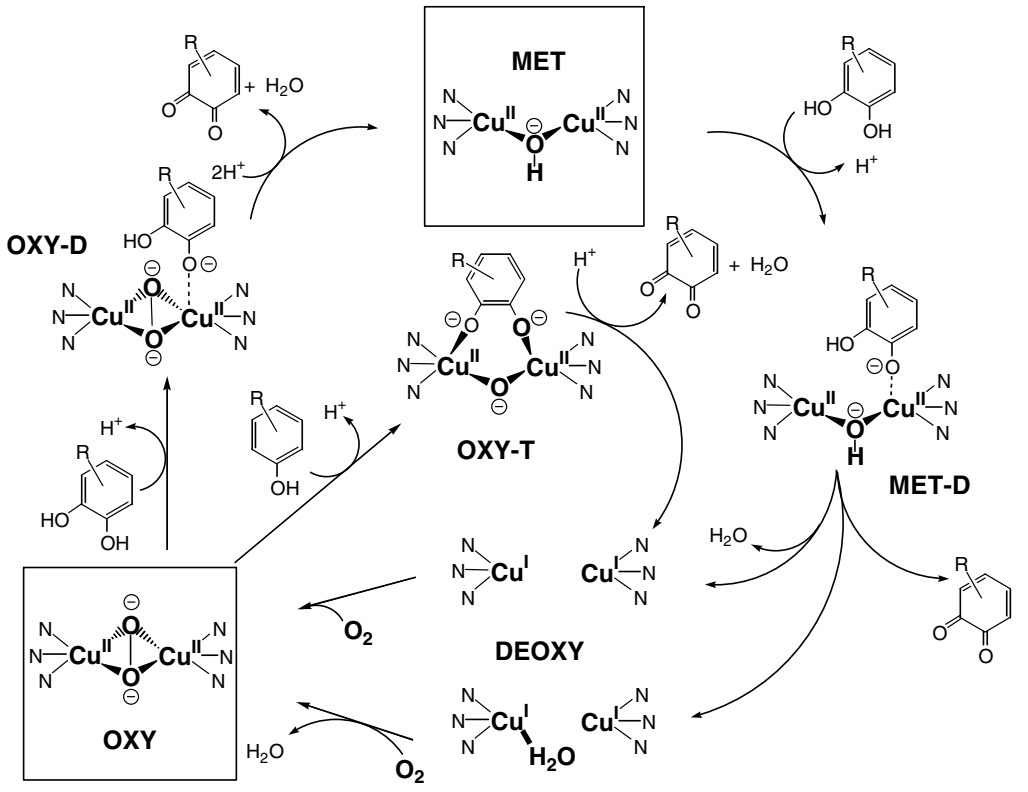


FIGURE 6.41 Reaction mechanism and cycling of polyphenol oxidase. Predominant, naturally occurring enzyme forms appear in boxes. OXY species are coordinated with two mole-atoms of O, while MET species are coordinated with $^-$OH. Some species have diphenol (D) or monophenol (T) bound at the active site. (Adapted and redrawn from Eicken, C., et al. (1999). *Curr. Opin. Struct. Biol.* 9:677–683 and Solomon, E.I., et al. (1996). *Chem. Rev.* 96:2563–2605.)

portion of the cycle starting with the DEOXY enzyme form, O_2 likely binds before the diphenol and forms a unique peroxide bridge (OXY form), receiving electrons from Cu^I–Cu^I.

Hydroxylation often exhibits a lag period since it requires the less abundant OXY-enzyme form and substituent groups on the substrate phenol ring may impede reactivity because of steric constraints of *o*-hydroxylation [119]. The hydroxylation sequence represents the inner cycle in Figure 6.41 and yields one mole H_2O per mole O_2 consumed. Monophenols appear to undergo both the sequential reactions of hydroxylation and oxidation in a single catalytic episode. Diphenols are activators of enzyme reactivity toward monophenols and reduce the lag period by allowing enzyme to cycle quickly from the MET to OXY forms (this feature is often expressed in Equation 6.47 as requiring an H-atom donor, BH_2 instead of $2H^+$). The reciprocal competitive inhibition of monophenols on *o*-diphenol oxidation and *o*-diphenols on monophenol *o*-hydroxylation is consistent with shared but partially divergent pathways of enzyme cycling for each activity. Low levels of H_2O_2 can activate tyrosinase by converting the MET form to OXY form; amounts in excess of this deactivate the enzyme, possibly by a crypto-oxy-radical generated by the binuclear Cu_2-peroxide complex, ultimately destroying the HIS ligands that secure Cu at the active site. Despite earlier reports of enzymes possessing only cresolase activity, it appears that all cresolase-type enzymes have catecholase activity with ratios of activity typically ranging 1:10 to 1:40 [149]. Most catecholase-type enzymes also have cresolase activity.

Enzymic browning occurs in shrimp and other crustaceans, and the defect is referred to as black spot. Hemocyanin, a copper protein involved in O_2 transport in crustaceans and closely related to tyrosinase, may have some involvement in the development of black spot. Laccases (EC 1.10.3.2) constitute another group of enzymes widespread in plants and fungi that oxidize diphenols, but do not exhibit cresolase-type activity. While they may contribute to enzymic browning reactions in foods, their properties are similar enough to *o*-diphenol oxidases (some differences in inhibitor sensitivities exist) that they will not be considered further here.

The role of phenol oxidases in plants is believed to be for defense against pests and pathogens [137]. The action of diphenol oxidases in plant tissue represents a classic decompartmentation mechanism of activation, since the enzyme is largely plastidic (chloroplasts and chromoplasts), can be as much as 95–99% latent, and may be complexed with an inhibitor (e.g., oxalate), and substrates are compartmented elsewhere (vacuoles or in specialized cells) or exist as precursors. The disruption of tissue can activate latent diphenol oxidases by acid and contact with substrates (from vacuoles), proteolytic processing of zymogen, or by various chemical activators, especially surfactants. The *o*-quinones produced by the enzyme reaction are reactive and can deactivate enzymes secreted by an invading organism, and the polymerization of *o*-quinones (melanosis) may also provide a physical barrier to infestation.

In foods, phenol oxidases are the cause of enzymic browning, which can be desirable in products such as raisins, prunes, cocoa beans, tea, coffee and apple cider. Phenol oxidases have also been shown to produce dityrosine cross-links and this may be beneficial where protein "texturization" is a desired outcome such as in gel formation and bread dough (gluten) conditioning. *In vivo*, tyrosinase has been implicated as being involved in betalain synthesis. However, in most fruits and vegetables, especially minimally processed products, enzymic browning is associated with color quality loss. The presence of phenol oxidases in grains, such as wheat, is correlated with lack of "whiteness" in noodles, a quality detriment.

Phenol oxidases in fruit and vegetative tissues exhibit optima in the general range of pH 4.0–7.0, and some substrates influence the pH optimum. Effects of pH are mediated by a single ionizable group that affects binding of substrate (K_M step) and not the catalytic (V_{max}) step or overall enzyme conformation. Temperature optima for phenol oxidases are in the range of 30–50°C, but temperature stability is comparatively high and characterized by half-lives of several minutes in the range of 55–80°C, depending on source. Thus, during thermal processing, ample opportunity exists for phenol oxidases to become activated, since temperatures ~60–65°C evoke cellular leakage (decompartmentation) and mixing of enzyme and substrate at elevated temperature.

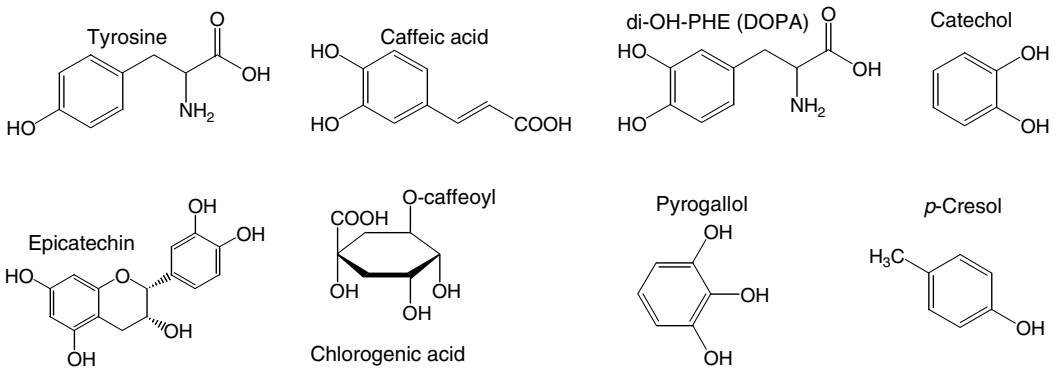
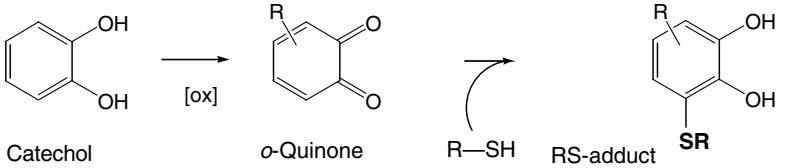


FIGURE 6.42 Polyphenol oxidase substrates.

Substrate preferences are dependent on enzyme source and isoform. Among the most common natural or endogenous substrates are caffeoyl-quinic acid, caffeoyl-tartaric and caffeoyl-shikimic acid derivatives, catechin, and those shown in Figure 6.42, where K_M values are in the general range of 0.5–20 mM. Some substrates are inhibitory at sufficiently high levels.

There is much interest in inhibiting enzymic browning and several strategies exist to do so. Dehydration, freezing, and thermal processing are effective as long as the time required to affect the process does not permit intolerable browning and textural changes related to quality retention. Other physical means include packaging in modified atmosphere for minimally processed foods, or coating of tissue sections with sugar syrups (especially for frozen products) or edible films to limit O_2 cosubstrate availability. This approach is made realistic by the K_M for O_2 being ~50 μM, and air-saturated water at 25°C is ~260 μM, providing opportunity for meaningful reduction in dissolved O_2 levels. The limitation for respiring products is that O_2 cannot be depleted to a level that evokes anaerobic metabolism that often yields off-flavors. While some phenol oxidases undergo reaction inactivation (by reaction with *o*-quinone), the thousands of enzyme turnovers that occur before inactivation happens limits the potential to exploit this feature as a means to control enzymic browning in foods.

Most popular are chemical treatments based either on inhibiting or deactivating enzyme, complexing native substrates, or reducing quinones back to *o*-diphenols and/or conjugating quinones in a manner that prevents melanin formation. For the latter strategy, chemicals that act only as reducing agents will delay browning only to the point where they are consumed, and then offer little further protection. Some reducing agents, especially thiols, can chemically conjugate quinones to form nonpolymerizing adducts, but this effect is also of limited duration since the thiol agents are consumed in the process:



(6.49)

Strategies revolving around enzyme inhibition have greater long-term effectiveness and include acidulants, enzyme inhibitors, chelating agents, and enzyme deactivators. Acidulants such as citric, malic, and phosphoric acids exploit the low pH sensitivity of enzyme action to the extent that addition of these acidulants can be used without other adverse effects. Inhibitors resembling native substrates may competitively occupy the phenolic binding site; such inhibitors appear in Figure 6.43.

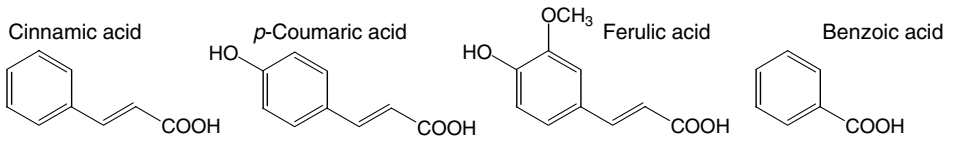


FIGURE 6.43 Polyphenol oxidase inhibitors.

Chelators, such as EDTA, oxalic acids, and citric acids (including juices that contain these organic acids, such as lemon and rhubarb), coordinate with copper at the active site, and there is evidence in some cases that a portion of the copper can be removed, although this is not necessarily required for inhibition. HIS binds copper quite tightly (log K_{assoc} of 10–18) and copper-chelating agents (log K_{assoc} of 15–19 for EDTA, and 4–9 for oxalate) may not be effective at copper removal from the enzyme active site. Other inhibitors coordinate to the active site copper and competitively inhibit activity; these inhibitors include halide salts, cyanide, CO, and some thiol reagents. Strategies to complex native substrates and limit their availability or access to enzyme reaction have focused on chitosan and cyclodextrin treatments. Prospective use of these agents may be limited to treating fluid products. Polyvinylpyrrolidone (PVP, insoluble form) is another phenolic-complexing matrix that is used primarily for research purposes in efforts to isolate phenol oxidases while minimizing the extent of browning that occurs during the process. However, this approach may diminish the nutritional value of juices as the phenols and related compounds are largely viewed as conferring health benefits (Chapter 12).

Reducing agents such as various sulfites, ascorbic acid, and cysteine (and the related tripeptide glutathione) have multiple effects on inhibiting enzymic browning. They may act by reducing *o*-quinones back to diphenols or chemically conjugating *o*-quinones, thereby delaying melanin formation. This effect would be of limited duration since reducing equivalents would become exhausted during sustained enzyme action. A more important effect of these agents appears to be irreversible, covalent inactivation of phenol oxidases, since enzyme activity is not fully restored by subsequent dialysis after extended preincubation in the absence of substrate [82]. These inhibitors appear to coordinate with active site copper, and undergo electron transfer reactions under aerobic conditions to yield "crypto" oxy-radicals (those not easily detected or identified) at the active site. These oxidizing species degrade the active site HIS ligands, inactivating the enzyme and likely releasing copper. The ability of inhibitory agents to function this way in disrupted tissues is based on kinetic factors, that is, how fast and competitively they bind to and inactivate the enzyme relative to how fast enzyme acts on substrates. Kinetics favoring the former favor inactivation of enzyme while kinetics favoring the latter would lead to depletion of added inhibitor (as a reducing or conjugating agent for *o*-quinones), limiting inhibitor effectiveness. Sulfites and thiol reagents are of longer-lasting effectiveness as browning inhibitors in disrupted tissues than ascorbic acid, and these distinctions correlate with a faster time frame of enzyme inactivation by the former group [82]. Tropolone and 4-hexylresorcinol are two more recently identified phenol oxidase inhibitors (Figure 6.44). They both resemble substrate and coordinate tightly with active site copper; these inhibitors are effective in the $\sim$1 μM range. 4-Hexylresorcinol was isolated from an extract of fig used as a ficin (protease) preparation. It is used primarily to control black-spot in crustaceans, as a replacement for sulfites, which are progressively being disallowed because of health-threatening responses of a proportion of humans, particularly asthmatics. Tropolone cannot be added to foods, but is useful in discriminating between browning caused by phenol oxidases and peroxidases. Another type of inhibitor of phenol oxidases are peptides in honey and corn seedlings that remain to be identified as well as various small cyclopeptides [137]. Kojic acid was identified from cultures of *Aspergillus* and *Penicillium* spp. as an effective phenol oxidase inhibitor, likely by coordinating to copper at the active site; however, its use may be limited to fermented foods using these organisms since historical data indicates toxicity in animals.

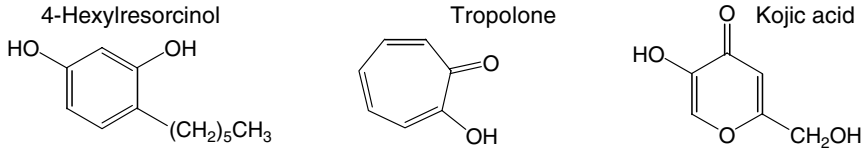


FIGURE 6.44 Polyphenol oxidase inhibitors.

6.5.2.2 Peroxidases [37,130]

Peroxidases are ubiquitous enzymes in plants, animals, and microorganisms, and are organized into plant (including microbes) and animal superfamilies. Plant peroxidases are most relevant to food biochemistry, and the various classes (families) of plant peroxidases include those of prokaryotic origin, secreted fungal peroxidases, and classical plant peroxidases. Plant peroxidases are glycosylated, monomeric, heme (protoporphyrin IX) proteins of 40–45 kDa mass comprised of two like domains, arising from gene duplication. Plant peroxidases are mostly soluble, with lesser proportions of membrane-associated and covalently bound forms, the latter types being released by cell wall degrading enzymes. Physiological roles of peroxidases include formation and degradation of lignin, oxidation of the plant regulator indole acetic acid (involved in ripening and associated catabolic processes), providing a defense to pest and pathogens, and removing cellular H_2O_2. Isoforms are classified as being acidic, neutral, and alkaline on the basis of isoelectric point. The neutral peroxidase-C of horseradish root (EC 1.11.1.7, donor:H_2O_2 oxidoreductase) is the most-studied member and consequently serves as a model peroxidase; its characteristics are generally applicable to other peroxidases. The general peroxidatic reaction catalyzed is

$$2AH\ (\text{electron donor}) + H_2O_2 \rightarrow 2H_2O + 2A^{\bullet} \tag{6.50}$$

The enzyme can exist in five oxidation states, with the resting state being the Fe^{III} form (Figure 6.45 [37]). Reaction with H_2O_2 occurs after docking near the heme iron, and HIS_{42} acts as a general base to "pull" an electron to yield the hydroperoxyl anion, a strong nucleophile that coordinates with Fe. The Fe-liganded HIS_{170} residue then acts as a general base to push electrons toward the peroxide and allows heterolytic O—O cleavage to yield H_2O as a leaving group (H^+ coming from HIS_{42} now acting as a general acid), yielding peroxidase compound I (Fe^{V}═O). Thus, a net $2e^-$ from heme Fe^{III} are used to reduce H_2O_2 and form H_2O. Two successive $1e^-$ (and H^+) transfer steps of two AH donors reverts the enzyme back to the resting state (completing the peroxidatic cycle), going through compound II (H^+–Fe^{IV}═O) and releasing another H_2O as a leaving group. Each of these steps are progressively slower relative to the rate of formation of Compound I. Peroxidases are most easily inhibited by chemicals that bind to the heme prosthetic group, the most common ones being cyanides, NaN_3, and CO, as well as some thiol compounds. However, use of such inhibitors is limited to characterizing peroxidases. Also, the general ambiguity regarding the role of peroxidase in food quality provides little justification for adding specific inhibitors.

Phenols (e.g., *p*-cresol, catechol, caffeic acid, and coumaric acid; Figures 6.42 and 6.43), ascorbic acid, NADH, and aromatic amines (e.g., *p*-aminobenzoic acid) are common electron donors for the conversion of Compound I to Compound II and back to ferric peroxidase. The $2A^{\bullet}$ resulting from the peroxidatic cycle can have various fates. If AH is ascorbic acid, then $2A^{\bullet}$ will yield one mole each of ascorbic acid and dehydroascorbate. If AH is guaiacol, then $2A^{\bullet}$ will undergo free radical addition (polymerization) to yield tetramers, and the attendant brown color provides the basis of

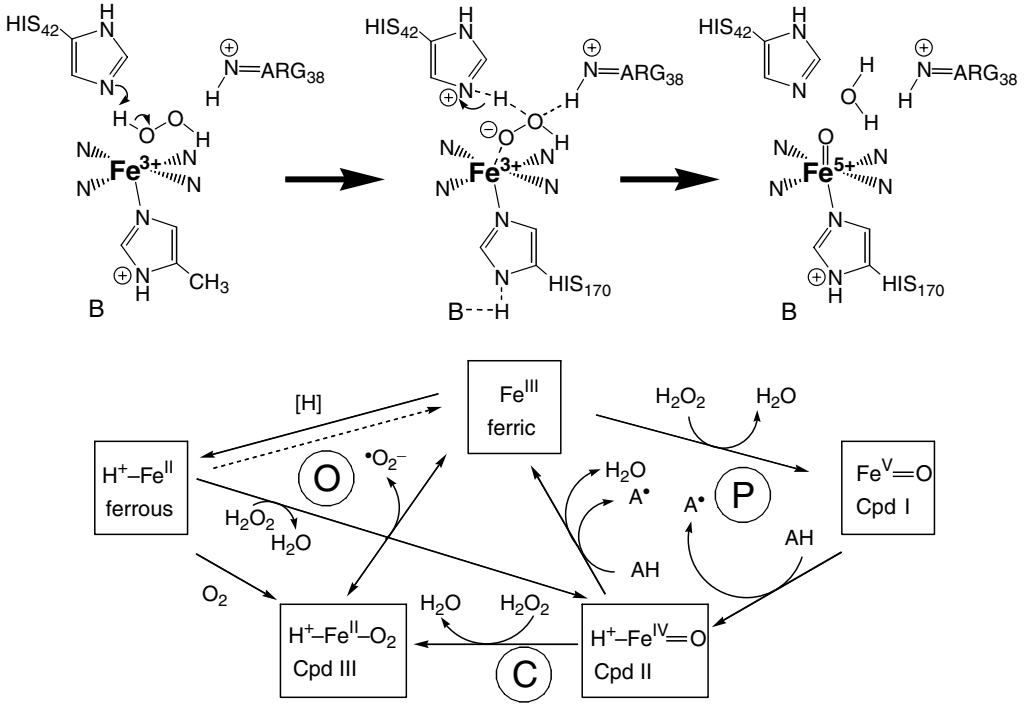
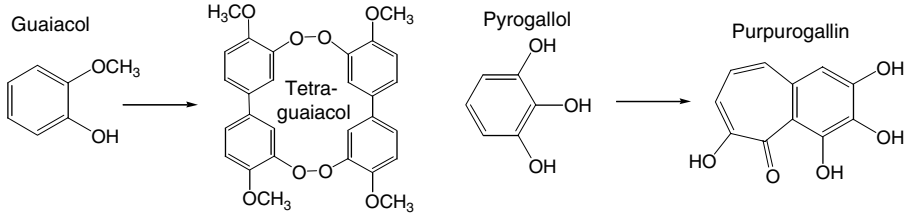


FIGURE 6.45 Reaction mechanism and cycling of peroxidase. **P** is peroxidatic cycle; **C** is catalatic cycle; and **O** is oxidatic cycle in bottom scheme. (Redrawn from Dunford, M.B. (1999). *Heme Peroxidases*, John Wiley & Sons, New York, p. 507.)

using guaiacol in the peroxidase assay widely used as a blanching efficacy indicator.



(6.51)

Pyrogallol is another substrate that undergoes free radical homocondensation reactions to yield a purple-colored dimer (purpurogallin). Tocopherol as AH can yield stable free radicals, whereas if tyrosine is used, the free radical adducts may condense to form dimers. Dityrosine cross-links in bread dough (gluten) may promote viscoelasticity and good baking qualities.

In the presence of excess H_2O_2, peroxidase will support a catalatic process (Figure 6.45) by reaction with a second mole of H_2O_2 to H_2O, forming compound III (H^+–Fe^{II}—O_2). Peroxidases exhibit maximum activity on AH donors at H_2O_2 levels of 3–10 mM, and these levels of use are important in peroxidase assays serving as blanching indicator tests. Assays using excess H_2O_2 will yield compound III, which is not recycled back to the resting state as efficiently, resulting in an underestimation of peroxidase activity.

There are other unique reactions exhibited by peroxidase. One involves NADH, which in the presence of trace H_2O_2 can react in the peroxidatic cycle as AH to yield 2 moles NAD•. NAD• may

have several fates and allow other reactions to occur:

$$NAD^{\bullet} + O_2 \rightarrow NAD + {}^{-}O_2^{\bullet} \quad (6.52)$$

$${}^{-}O_2^{\bullet} + 2H^{+} \rightarrow H_2O_2 \quad (6.53)$$

$$NAD^{\bullet} + \text{ferric peroxidase} \rightarrow NAD + \text{ferrous peroxidase} \quad (6.54)$$

$$\text{Ferrous peroxidase} + O_2 \rightarrow \text{oxyperoxidase (compound III)} \quad (6.55)$$

$$\text{Oxyperoxidase} \rightarrow \text{ferric peroxidase} + {}^{-}O_2^{\bullet} \text{ (then Equation 6.53 may follow)} \quad (6.56)$$

Thus, using NADH, peroxidase has the ability to generate its own cosubstrate (H_2O_2) when only trace levels exist, making use of both the peroxidatic and oxidatic cycles.

Other types of peroxidase-associated activities, oxidation and hydroxylation, are indirect effects of peroxidase reactivity. The sequence using NADH as AH in the peroxidatic and oxidatic cycles illustrates how peroxidase action can yield reactive oxygen and oxy radicals. Such species may cause oxidation reactions. Oxidation reactions can occur if a cosubstrate yields $A^{\bullet}$ species that can abstract H atoms from other components. Such a sequence can initiate other free radical reactions that could possibly lead to polymeric derivatives being formed from phenolic components, reminiscent of phenol oxidase-mediated browning. Thus, reaction of peroxidase with one phenolic substrate may cause indirect (chemical) oxidation of another, potentially obscuring an evaluation of direct peroxidase action on components in a mixed system such as foods. Phenolic peroxidase substrates that yield O_2-reactive $A^{\bullet}$ will also form ${}^{-}O_2^{\bullet}$ and H_2O_2, which can further mediate oxidation reactions. Thus, how much of a role peroxidases plays in browning and other discoloration processes in foods has remained enigmatic. Some of the more recent claims of peroxidase involvement in browning are based on correlative associations of peroxidase activity and levels or incidence of browning; such observations remain short of establishing cause and effect.

Plant peroxidases often exhibit pH optima in the range of pH 4.0–6.0, although the pH range for forming compound I is very broad with pK_a values of ~2.5 and 10.9 to mark the pH transitions in activity. The acidic transition is conferred by the HIS_{42} residue, the pK_a of which can vary between 2.5 and 4.1, depending on medium composition. This is an unusually low pK_a for HIS that must first act as the conjugate base, and it is brought about by multiple H-bonding networks that serve to facilitate H^{+}-dissociation. The overall pH optima for peroxidase reactions relates to the steps that utilize AH to recycle ferric peroxidase in the peroxidatic cycle. AH species are H-donors (not just e^{-} donors), and thus, must be protonated (if it has a dissociable H^{+}) to serve as substrate, and pH optima are often substrate dependent.

Peroxidases are among the most ubiquitous and heat stable enzymes in plant tissues; these characteristics favor their use as blanching indicators. The rationale is that if endogenous peroxidase activity is destroyed, all other quality-deteriorative enzymes must be as well. The limitation of this strategy is that excessive thermal processing is often applied and this may compromise quality in various others ways (e.g., texture, nutrition, component leaching). However, until other specific enzymes are identified as being the most heat stable among those having direct impact on quality of blanched (and the frozen) vegetables, and are easy to assay, peroxidase will remain the blanching indicator of choice. Temperature effects on peroxidases vary with the host tissue. Generally, optimal temperature for activity is modest, ranging from 40°C to 55°C. Thermal stability is quite high, and depending on source, complete inactivation may require a several-minute exposure at 80–100°C for appropriately sized, intact portions of vegetable tissues. The heme prosthetic group, glycosylation, four disulfide linkages, and the presence of 2 moles Ca^{2+} with likely participation in salt bridges are factors responsible for peroxidase thermal stability. Thermal stability generally decreases as pH decreases in the range of pH 3–7 and in the presence of increasing ionic strength. Regeneration of peroxidase activity occurs in the range of pH 5.5–8.0, following short durations of thermal processing, such as blanching. Regeneration is believed to involve reconstitution of the heme at the active site

that was lost during initial deactivation. More extensive heating, such as retorting, diminishes the propensity for regeneration of active enzyme because of more extensive conformational changes and covalent reactions. However, the release of free heme into the medium may provide for catalysis of oxidative reactions and such processes have been implicated as causing off-flavors in canned vegetables. Other reactions catalyzed by peroxidase that impact quality in foods include formation of phenoxy radicals that indirectly oxidize lipids and the direct oxidation of capsaicin, the pungent principle of peppers.

While the role of peroxidase in enzymic browning remains open to question, it has been conclusively shown that peroxidase can destroy some pigments, particularly betalains in table beet roots. Peroxidase has also been implicated in the bleaching of chlorophyll under specific conditions.

6.5.2.3 Other Oxidoreductases [37]

Lactoperoxidase is the peroxidase in milk, and belongs to the animal superfamily of peroxidases. It is a 78 kDa mass glycoprotein monomer, containing Ca^{2+} and a modified protoporphyrin IX that is covalently bound. Lactoperoxidase has properties similar to horseradish peroxidase-C in terms of H_2O_2 reactivity and cycling through peroxidase forms. Lactoperoxidase is particularly distinct from peroxidase-C in that it is more reactive with halides (especially I^-) and related species. Of particular interest is the ability to react with thiocyanate (SCN^-), which is normally present in milk, as AH in the peroxidatic cycle where

$$2SCN^- + Enz\text{–}(Fe^{V}{=}O) \rightarrow 2SCN^{\bullet} + Enz\text{–}(Fe^{III}) \rightarrow SCN^- + HOSCN + H^+ \qquad (6.57)$$

The hypothiocyanous acid and conjugate base (pK_a 5.3) hypothiocyanite ($OSCN^-$) are antimicrobial agents. Thus, addition of small amounts of H_2O_2 (and also SCN^-, if not abundant) to milk affords a "cold-pasteurization" process that reduces microbial load in raw milk and this is an important option in (sub)tropical climates where ready access to refrigeration may not be available. The enzyme-generated $OSCN^-$ is more effective than adding exogenous chemical, perhaps, because lactoperoxidase adsorbs to surfaces and particulates and may afford $OSCN^-$ generation in proximity to microorganisms.

Catalase (EC 1.11.1.6) is a tetrameric heme enzyme that is widespread in nature and is related to peroxidases. Its principal role is to detoxify cells of excess H_2O_2 as the enzyme degrades H_2O_2 to H_2O plus $\frac{1}{2}O_2$. Catalase is rather heat stable and has been considered as a blanching indicator enzyme. It is easy to assay, by taking a small filter paper disk, dipping it into a homogenate of blanched vegetable, and then placing the disk into a test tube of dilute H_2O_2. A positive test for residual catalase is indicated by the disk floating to the surface, buoyed by small, adherent O_2 bubbles formed by any active enzyme absorbed on the disk.

6.5.3 Enzymes Related to Flavor Biogenesis

6.5.3.1 Lipoxygenase [14,22,102,141]

The role of lipoxygenases in foods and food quality continues to be evaluated, despite these enzymes being characterized over 70 years of prior study. Some of the earliest descriptions referred to "lipoxidase" and "carotene oxidase" activities. Lipoxygenases (and related oxygenases) are widespread and found in plants, animals, and fungi, while once they were believed to exist exclusively in the plant kingdom. Lipoxygenase mechanism and the basis of reaction selectivity were featured earlier in this chapter. This section will focus on the multiplicity of reaction and ancillary pathways of fatty acid transformation and associated roles of lipoxygenase-mediated processes in food quality. Lipoxygenase action may be desirable or undesirable, depending on the specific food material and

the context in which it is used, and many examples beyond what appears forth are provided in various reviews [22,141].

Lipoxygenase has long been known to cause quality defects in processed vegetables that have not been sufficiently thermally processed to destroy the enzyme. Legumes (snap beans, soybeans, peas) are particularly susceptible to the development of oxidative rancidity because of high lipoxygenase levels (Table 6.12 [45,50,63,72,135]). The diversity of lipoxygenase-mediated reactions can be accounted for by the reaction cycle extending beyond that required to illustrate mechanism (recall Figure 6.8). The anaerobic cycle encompasses reactivity of the enzyme in the absence of O_2 or in O_2-starved media; this portion includes the peroxide activation of enzyme resting state (Fe^{II}) to the active state (Fe^{III}), sometimes referred to as "lipoperoxidase" activity (Figure 6.46 [56,141,144]). As a result of this activation, an oxy-radical species ($XO^{\bullet}$) is released, which can propagate free radical reactions; this cycle may continue in the absence of O_2 through which fatty acid radicals ($L^{\bullet}$) may be formed and released. In cases where $XO^{\bullet}$ is derived from a polyunsaturated fatty acid, it may undergo intramolecular rearrangement and form reactive epoxides. Thus, many lipoxygenases cause secondary, free radical cooxidation reactions when the anaerobic cycle is operative. When O_2 is abundant, the normal reaction mechanism occurs as explained earlier (Figure 6.8). Some lipoxygenases have lesser affinities for fatty acid and reaction intermediates, and the hydroperoxyl radical ($LOO^{\bullet}$) may dissociate prematurely through the "low affinity loop" before the normal catalytic cycle is completed (Figure 6.46). This requires that the enzyme become reactivated by peroxide in the anaerobic cycle. The substrate affinity of lipoxygenase isoforms 3 of pea and soybean seeds is 20-fold less than the other respective seed isoforms [7,56]. Thus, pea and soybean isoforms 3 are primarily responsible for yielding $LOO^{\bullet}$ to cause further fatty acid autoxidation and cooxidation reactions through the evolution of reactive oxygen species, including singlet oxygen ($^1O_2$) during the aerobic cycle (most isoforms cause cooxidation only in the anaerobic cycle). The aerobic and anaerobic cycles constitute alternative pathways of enzyme cycling, and both of these pathways may operate simultaneously. The precise O_2 level is not always the sole determinant of the preferred pathway of enzyme cycling. Kinetic characteristics for each step, relative levels of enzyme, substrate and intermediates, as well as the enzyme microenvironment, influence the degree to which each pathway is evoked.

Lipoxygenases from various food sources differ in isoform profiles, optimum pH, and reaction regio- and stereoselectivity (Table 6.12). Lipoxygenases are "soluble" enzymes but different isoforms are found in different cellular compartments, reflecting their unique purpose and role in fatty acid transformation in the host tissue [43]. Soybean seed lipoxygenases are the most studied and historically comprised the basis for classification [7]. Lipoxygenase isoform 1 is the most abundant in soybean seed and is unusual in its alkaline pH optimum. This feature and the 13*S*-product stereoselectivity led it to be classified as a "Type I" lipoxygenase. Soybean isoforms 2 and 3 have more neutral pH optima and exhibit less product selectivity; these general features comprised the historical basis for the "Type II" classification. It is now clear that most lipoxygenases have near-neutral pH optima and differ widely in degree of product selectivity, rendering the original "Type" classification of limited usefulness. Even the classification on the basis of regioselectivity of oxygenation of arachidonic acid (e.g., 5-LOX) described earlier is yielding in favor of classification on the basis of structural similarities. A selective survey of plant lipoxygenases (Table 6.12) reveals that many are regioselective for oxygenating C9 of linoleic (or linolenic) acid to yield the *S*-configured hydroperoxide (LOOH). Some lipoxygenases (especially soy isoforms 2 and 3, and pea seed isoforms) are lacking in regio- and stereoselectivity. This property is associated with a reduced affinity for fatty acid substrate during the reaction sequence [7,56]. If $L^{\bullet}$ is released prematurely, chemical combination with molecular O_2 will be random (nonselective) leading to *rac*-LOOH mixtures, whereas oxygenation while substrate is at the enzyme active site will exhibit regio- and stereobias (Figure 6.46).

As indicated earlier, isoforms causing cooxidation reactions yield multiple autoxidation products, including aldehydes and ketones (carbonyls) produced through a free-radical mechanism.

TABLE 6.12
Properties of Selected Lipoxygenases and Hydroperoxide Lyases

Lipoxygenase Source (Isoform)	Relative Activity	Opt. pH	Lipoxygenase Specificity 9:13, *S*/*R*	Hydroperoxide Lyase Specificity	Dominant Compounds in Host Tissues
Soybean seed (1)	4200	9.0	4:96 13*S* (pH 9) 23:77 13*S* (pH 6.6)	*S*-13-LOOH (low levels)	*n*-Hexanal, hexanals, off-flavors
(2)		6.5	50:50 9*R* ≥ 9*S*		
(3)		7.0	65:35 *R* ~ *S*		
Corn germ	—	6.5	93:7 9*S*	(Trace/low levels)	*n*-Hexanal, off-flavors (ketols in corn seed)
Pea seed (3 isoforms)	1800	6.6	67:33 *R* ~ *S* (pH 6.6) 59:41 13*S*, 9*R* (pH 9)	(Trace/low levels)	Off-flavors
Potato tuber	4600	5.5	95:5 9*S*	9/13-LOOH	*trans*-2-*cis*-6-nonadienal
Tomato fruit (3 isoforms)	360	5.5	96:4 9*S*	13-LOOH (CYP74B)	*trans*-2-hexenal, *cis*-3-hexen-1-ol, *n*-hexanal
Cucumber fruit	30–120	5.5	75:25	9,13-LOOH	*trans*-2-*cis*-6-nonadienal
Green pepper fruit	300	5.5–6.0	Lacking definitive evaluation	13-LOOH (CYP74B)	*cis*-3-hexenal, *trans*-2-hexenal, *n*-hexanal
Pear fruit	Trace	6.0	95:5	9-LOOH	*trans*-2-*cis*-6-nonadienal
Apple fruit	<120	6.0–7.0	15:85	13-LOOH	*n*-hexenal, *trans*-2-hexenal
Mushroom	—	8.0	10:90 13*S*	*S*-10-LOOH	1-octen-3-ol, 1-octen-3-one
Tea leaves	—	6.5	16:84 13*S*	*S*-13-LOOH	*trans*-2-hexenal, *cis*-3-hexenal, *n*-hexanal

Source: Galliard, T. and Chan, H.-W.-S. (1980). In *The Biochemistry of Plants. A Comprehensive Treatise, Volume 4, Lipids: Structure and Function*, Stumpf, P.K. (Ed.), Academic Press, New York, pp. 131–161; Grosch, W. (1982). In *Food Flavours. Part A. Introduction*, Morton, I.D. and MacLeod, A.J. (Eds.), Elsevier Scientific Publishing, New York, pp. 325–398; Kuribayashi, T., et al. (2002). *J. Agric. Food Chem.* 50:1247–1253; Matsui, K., et al. (2001). *J. Agric. Food Chem.* 49:5418–5424; and Vliegenthart, J.F.G. and Veldink, G.A. (1982). In *Free Radicals in Biology*, Pryor, W.A. (Ed.), Vol. V, Academic Press, New York, pp. 29–64; Pinsky, A., Grossman, S. and Trop, M. (1971). *J. Food Sci.* 36:571–572.

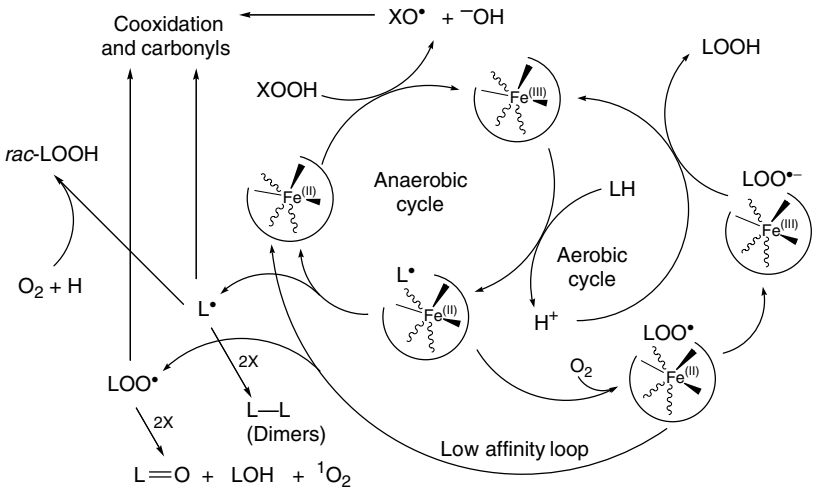
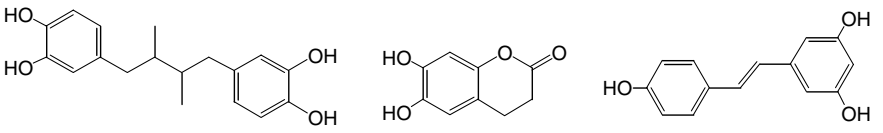


FIGURE 6.46 Reaction pathway and cycling of lipoxygenase. (Adapted and redrawn from Hughes, R.K., et al. (1998). *Biochem. J.* 333:33–43; Whitaker, J.R., et al. (Eds.) (2003). *Handbook of Food Enzymology*, Marcel Dekker, New York; and Wu, Z., et al. (1999). *J. Agric. Food Chem.* 47:4899–4906.)

Cooxidation also bleaches carotenoids, and while this may destroy (pro)nutrients, it is a useful and desirable outcome in whitening of bread dough and related finished baked goods (oxy-radical generation may also improve dough tensile and viscoelastic properties). Both soy and pea seed flours (as well as those from potato and chick pea) may be added to bread dough for their carotene-bleaching and dough-improving effects, since wheat lipoxygenase has low bleaching activity. Lipoxygenases from tomato and green pepper fruit are also capable of cooxidizing carotenoids. Many other plant food sources have multiple lipoxygenase isoforms, including most cereals and grains, as well as snap beans.

Thus, the oxidative rancidity defect brought about by lipoxygenase can be attributed to two phenomena (covered in detail in Chapter 4). One is the oxidation of linoleic and linolenic to LOOH, and the ensuing chemical decomposition into various aromatic aldehydes and ketones. The second is the direct enzymic production of fatty acid radicals released to the food matrix that further initiate and propagate cooxidation and free radical autoxidation reactions. *n*-Hexanal confers a beany flavor and is used as a general index of the degree of fatty acid oxidation. Among the sources of lipoxygenase listed (Table 6.12), soybean, corn, and peas are most prone to developing rancidity derived from lipoxygenase action. This requires corn and peas to be blanched to at least a lipoxygenase deactivation end-point prior to freezing and storage; in soybeans, lipoxygenase action must be destroyed or attenuated before freezing (by blanching), grinding into flour (by drying), or refining into oil and protein isolates.

The rather subtle differences between lipoxygenase isoforms have been exploited in efforts to manage food quality. Isogenic strains of soybean seeds, lacking in certain lipoxygenase isoforms, have been assessed for their propensity to cause rancid flavors in beans, soybean oil, and composite foods (bread). For homogenized soybean seeds, lipoxygenase-2 appears responsible for producing the greatest levels of *n*-hexanal [55]. The presence of either isoforms 1 or 3, or both, reduced the capacity of isoform-2 to produce *n*-hexanal, suggesting that the fates of fatty acid hydroperoxides are dependent on the isoform that produced it. When flour from soybean strains lacking in specific isoforms was used in bread dough, isoform-1 was associated with greatest increases in bread volume and isoform-2 was associated with greatest increases in dough strength and viscoelasticity [31]. Isoform-2 was also associated with greater levels of objectionable volatiles, and this is why legume flours are added at $<1\%$ in bread doughs. These examples show how knowledge of even subtle differences in enzyme action may lead to new strategies to produce foods and manage quality.



Nordihydroguaiaretic acid Esculetin Resveratrol

FIGURE 6.47 Lipoxygenase inhibitors.

Lipoxygenases and related oxygenases (cyclooxygenases) occur in animal tissues, and muscle systems are most relevant to foods [14,22]. Animal lipoxygenases are essentially identical to plant and fungal lipoxygenases in terms of structure and mechanism. One major difference is that arachidonic acid, and longer-chain, higher unsaturates are the natural substrates for animal lipoxygenases, although they are also active on linoleic and linolenic acid. In fresh fish, endogenous lipoxygenase action is known to form desirable flavors (Chapter 10), but information remains lacking on the relationships between animal lipoxygenases and food quality.

The most effective means to prevent negative consequences of lipoxygenase action is thermal processing to deactivate the enzyme, with pH adjustment being a secondary approach. Many compounds have been identified as inhibitors of the enzyme, and those with typical phenolic-antioxidant properties quench secondary oxidation reactions with little direct effect on the enzyme. Only a few inhibitors have been consistently identified to directly inhibit lipoxygenase (Figure 6.47), and these include nordihydroguaiaretic acid, catechols, and esculetin (all at $\sim$10 μM), which coordinate with active site Fe and/or reduce it to the inactive Fe^{II} state. Also, resveratrol ($\sim$10 μM) and $SnCl_2$ (5 mM) are competitive inhibitors.

6.5.3.2 Hydroperoxide Lyase and Related Enzyme Transformations [12,135]

The decomposition of fatty acid LOOH (derived from lipoxygenase action) by *nonspecific*, chemical reactions to yield carbonyls that confer rancidity is minimized by the presence of enzymes that *specifically* direct LOOH transformation toward other derivatives. In many fruit and vegetative tissues, this alternative pathway is evoked by hydroperoxide lyases, leading to the accumulation of a limited set of degradation products of 6, 9, and 12 carbons of defined composition (Figure 6.48 [12,43,135]; Table 6.12). The general sequence of events is the liberation of fatty acid from intact glycerolipid by lipid acyl hydrolase, dioxygenation of fatty acid into 9/13-hydroperoxides by lipoxygenase, cleavage of the 9/13-hydroperoxides by hydroperoxide lyase, and then possible isomerization and final conversion of aldehydes to alcohols by alcohol dehydrogenase activity. The existence of this pathway was first inferred for the origin of banana flavor and the enzymology was definitively established for cucumber and tomato fruit [45,50,135].

The species-specific pathway of transformation of fatty acids into desirable flavors derives from the combined effects and specificity of both lipoxygenase and hydroperoxide lyase, and the relative abundance of auxiliary enzymes. For example, for tomato fruit, even though fatty acid 9-LOOH is the dominant product of lipoxygenase action (Table 6.12), the specificity of the hydroperoxide lyase (9:13-LOOH at 1:62 relative rates) dictates that the C6 and C12 fragments are mostly produced in disrupted tissue. In contrast, cucumber fruit hydroperoxide lyase is rather nonspecific (9:13-LOOH at 2:1 relative rates), and the dominance of C9 fragments from directed fatty acid oxidation and fragmentation is dictated largely by lipoxygenase selectivity. Examples listed in Table 6.12 can be grouped as forming principally cucumber-type flavors (nonadienal species), or tea-leaf-like flavors and rich in floral/fruity flavors conferred by hexenal/hexanals/hexenols. The reason why all examples within a group (C9 or C6 accumulators) do not have the same overall flavor is dependent on several other factors involved in flavor biogenesis. Two factors involve differences in the levels of the composite enzymes in the pathway, as well as ratios of linoleic and linolenic acid substrate

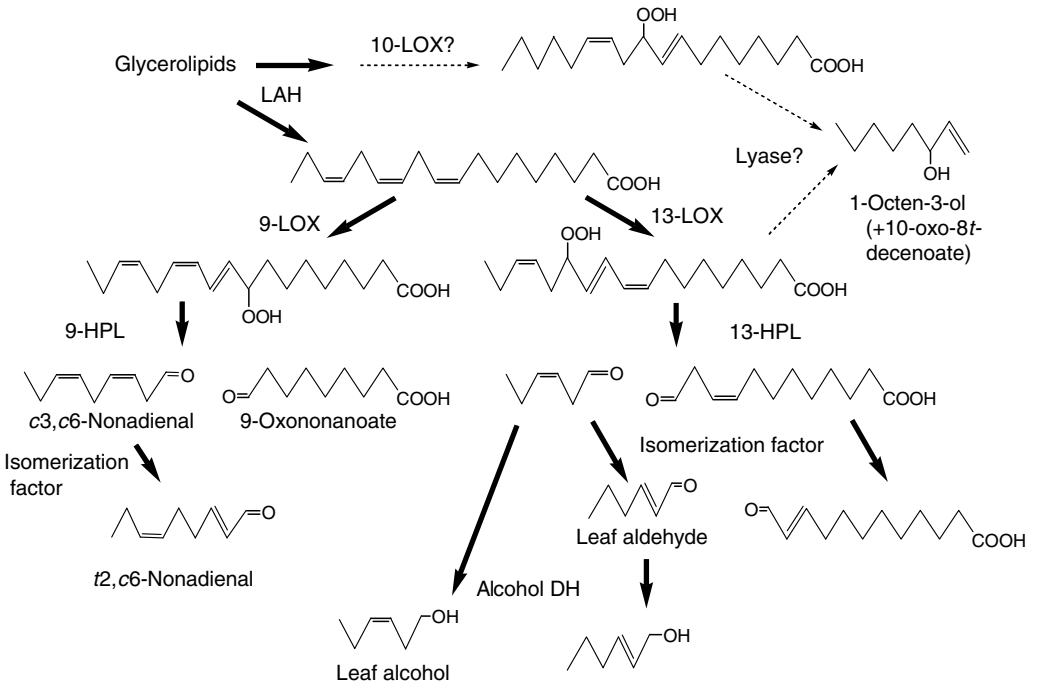


FIGURE 6.48 Coordinated lipoxygenase (LOX), hydroperoxide lyase (HPL), and auxiliary enzyme transformation of fatty acids to yield "green" notes and flavors. (Adapted from Blée, E. (1998). *Prog. Lipid Res.* 37:33–72; Fuessner, I. and Wasternack, C. (2002). *Annu. Rev. Plant Biol.* 53:275–297; and Vliegenthart, J.F.G. and Veldink, G.A. (1982). In *Free Radicals in Biology*, Pryor, W.A. (Ed.), Vol. V., Academic Press, New York, pp. 29–64.)

liberated by lipid acyl hydrolase action. The pathway (Figure 6.48) is shown for linolenic acid, but the same reactions may occur for linoleic acid to yield analogous products of different flavor character, and differences in enzyme selectivity for these fatty acids will impact final product composition. Differences in tissues among the auxiliary enzymes will also dictate final product composition. While evidence of specific isomerizing enzymes has been obtained for cucumber, flaxseed, wheat germ, barley, and soybean [45,135], these isomerizations may be largely nonenzymic in nature, and the isomerization factor remains ambiguous [12]. Hydroperoxide isomerase (originally listed as EC 5.3.99.1, but deleted in 1992) is now accounted for by the combined action of hydroperoxide dehydratase (allene oxide synthase, EC 4.2.1.92) and allene-oxide cyclase (EC 5.3.99.6). Little is known of the alcohol dehydrogenase involved, whether there is an isoform specific for this pathway, or if basal cellular alcohol dehydrogenase is involved [12]. Each tissue also has other flavoring agents, conferred by other metabolic or enzyme pathways that contribute to or even dominate the overall flavor character of the food sources listed in Table 6.12.

Hydroperoxide lyases (classified as cytochromes, CYP74B and CYP74C) are likely tetramers of 55–60 kDa monomeric units and are membrane bound (plastidic) in tissues [43]. They differ from other cytochromes in that they do not require O_2 and NAD(P)H; instead they utilize the fatty acid LOOH as both substrate and oxygen donor in forming new C—O bonds. They have a high catalytic turnover rate of 10^3 per second [49]. It is not surprising that many lipoxygenase inhibitors (Figure 6.47) also inhibit hydroperoxide lyase since both enzymes bind fatty acid chains. In addition to the four tetrapyrrole ligands, the Fe is coordinated with a CYS [43]. The enzymes from pepper, tomato, and guava fruits are highly 13-LOOH specific and are placed in the CYP74B subfamily, whereas those from cucumber and melon act on both 9/13-LOOH and are placed in the CYP74C subfamily. Other hydroperoxide lyases remain to be fully characterized and classified.

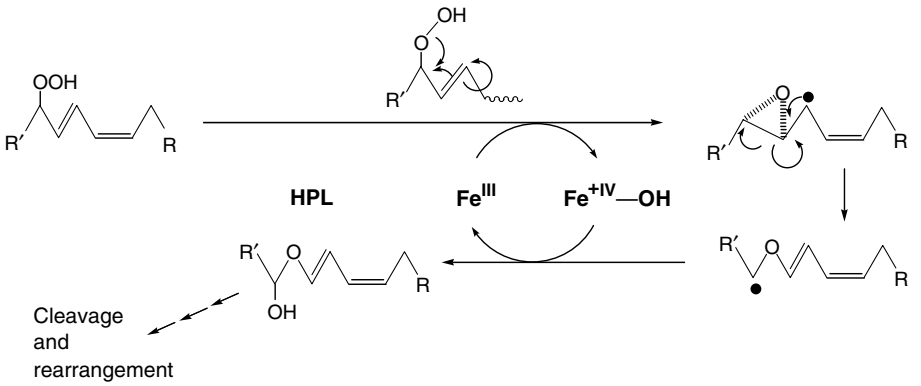


FIGURE 6.49 Reaction mechanism of hydroperoxide lyase. (Adapted and redrawn from Gretchkin, A.N. and Hamberg, M. (2004). *Biochim. Biophys. Acta* 1636:47–58.)

Particularly intriguing is the enzyme system in mushrooms, which forms C10 and C8 fragments from dioxygenated linoleic and linolenic acids (Table 6.12; Figure 6.48). Initially the presence of a 10-LOX was proposed, but it has since been established that mushroom lipoxygenase dioxygenates the C13 site [63]. However, the linoleate 10-LOOH isomer is formed in mushroom [4], and mushroom protein preparations can cleave the 10-LOOH derivative to C10 oxo-acid and 1-octen-3-ol. Thus, major questions remain about the nature of the enzyme activities involved in mushroom volatile generation from linoleic and linolenic acids.

The mechanism of action of hydroperoxide lyase (and the other CYP74 family members) has recently been proposed from studies on the guava fruit enzyme. The homolytic mechanism involves an epoxyallylic radical leading to a hemiacetal, which lyses to yield fragmentation products (Figure 6.49 [49]). This mechanism probably applies to all hydroperoxide lyases, and it is consistent with the established mechanism of cytochrome P450s.

Efforts to apply knowledge of the lipoxygenase–hydroperoxide lyase pathway to genetically modified fruits have shown mixed results. Introducing a $\Delta 9$ desaturase (to enhance fatty acid substrate) and alcohol dehydrogenase in tomato fruit both enhanced flavor, whereas suppressing lipoxygenase and overexpressing a 9-hydroperoxide lyase had no effect [72]. Commercial opportunities are currently employing highly active 13-specific lipoxygenase and fatty acid 13-LOOH hydroperoxide lyase in combination with an inexpensive source of linolenic acid to produce the "green note" flavors conferred by the 6-C aldehydes and alcohols, a global market estimated at more than US $40 million annually.

6.5.3.3 Biogenesis of Other Lipid-Derived Flavors [106]

Alcohol acyltransferase (EC 2.3.1.84) is responsible for the emanation of aromatic esters in many climacteric fruits, especially during the ripening phase whereupon their tissue levels increase [106]. This enzyme exists in fruits such as apples, strawberries, bananas, melons, and olives, among others, and also in yeast and fungi. The reaction catalyzed is

$$S\text{-Acyl–coenzyme A} + \text{alcohol} \leftrightarrows \text{Acyl–ester} + \text{Coenzyme A–SH} \qquad (6.58)$$

The typical profile of esters formed in ripening fruit by this enzyme includes acetate and butanoate esters of methyl- and ethyl-branched, phenylethyl, or *n*-alcohol groups, typically of 2–8 carbons.

Other lipid flavors in foods may arise from other pathways. Lipases have been considered as mediators of volatile ester formation, through reverse hydrolysis reactions. There is no evidence of

this in fruit tissues, but it occurs to some extent in yeast fermentations, and low levels of fruity flavors may be mediated by fermentative organisms and contribute to overall flavor of aged, fermented foods such as cheese. Terpenoid biosynthesis in plants typically used as herbs and spices occurs through a multistep, complex biosynthetic pathway from isoprene units [106]. A lipoprotein lipase (LPL, stimulated by lipoprotein) occurs in milk at 1–2 mg L^{-1} [141] and may give rise to "spontaneous rancidity" if milk is handled poorly before pasteurization.

6.5.3.4 Origin and Control of Pungent Flavors and Other Bioactive Effects

6.5.3.4.1 Myrosinase Transformation of Glucosinolates [5,75]

Plants of the family Brassicaceae, notably cabbage, broccoli, cauliflower, turnip, kale, Brussels sprouts, radish, mustard, and wasabi, are known for their pungency. Upon tissue disruption, conditions become favorable for reaction between the enzyme myrosinase (EC 3.2.1.147 [formerly 3.2.3.1], thioglucoside glucohydrolase) and a diverse set of odorless glucosinolate substrates, setting off a "mustard oil bomb." Although subject to debate, the prevailing view is that specialized cells (idioblasts) called "myrosin cells" contain myrosinase and these are adjacent to S-cells (sulfur-rich) that contain both glucosinolates (as high as 100 mM) and ascorbate in vacuoles [5]. When tissue is disrupted, the ascorbate becomes diluted to the 1–2 mM level that activates the enzyme as it mixes with glucosinolate substrates. Myrosinases are 10–20% glycosylated, and they exist as multiple isoforms of 65–70 kDa monomeric mass with structures stabilized by three disulfide bridges and by Zn^{2+}; they may also exist as homooligomers or in complexes with other proteins. Myrosinases exhibit optimum activity in the ranges of pH 4–8 and 40–75°C, depending on source.

Myrosinase is a retaining enzyme of family 1 of the glycosyl hydrolases (Section 6.3.2) and is unique on two accounts. It hydrolyzes β-D-thioglucosides (and not *O*-glycosides), and it has only one of the usual two ASP/GLU catalytic residues [17]. The enzyme has a hydrophobic binding pocket (mustard seed enzyme as reference: $PHE_{331,371,473}$, ILE_{257}, TYR_{330}) to host the R-groups of the glucosinolate, which are mostly alk(en)yl chains that may be branched or substituted with S, S═O, keto, or hydroxyl groups [75]. Several residues (GLU_{464}, GLN_{39}, HIS_{141}, ASN_{186}) provide H-bonding to the glucose with TRP_{457} providing the hydrophobic platform for stacking of the pyranose ring [17]. GLN_{409} and $ARG_{194,259}$ coordinate with the sulfate groups of the substrate. GLU_{409} constitutes the enzyme carboxylate nucleophile to displace the *S*-aglycon (a good leaving group) and form an enzyme–glucose covalent intermediate (Figure 6.50 [17,18,75]). The rate-limiting step is the release of the glucose, and in other glycosyl hydrolases, a second conserved carboxylate (GLU or ASP) serves this purpose by activating water as a nucleophile. This function is missing and some assistance to activate water to displace glucose (and retain the β-configuration) may be afforded by H-bonding with GLN_{187}. A long-recognized activating effect of ascorbic acid on myrosinase (of few- to several hundred-fold on V_{max}) is now believed to be conferred by ascorbate acting as an "external" cofactor [18]. Ascorbate binds to the enzyme after the aglycon is released (they share the same binding site) and ascorbate seems to function by activating nucleophilic water to displace the glucose. The distance between the GLU_{409} and ascorbate is ~7.0 Å, greater than the 4.5 Å distance between catalytic GLU/ASP of the typical retaining glycosyl hydrolase, but this may be necessary to accommodate the bulkier ascorbate residue. Maximum activation of ascorbate occurs at 0.5–1.5 mM; excess ascorbate competes with glucosinolate binding and impedes enzyme cycling.

The fate of the hydrolyzed glucosinolate also depends on compositional factors and the nature of the glucosinolate structures, some of which are shown in Figure 6.51 [73]. Under conditions of pH that typically prevail in vegetative tissues, the isothiocyanate derivatives are formed (Figure 6.50). In the presence of Fe^{2+} and an epithiospecifier (nonenzymic) protein, epithionitriles may accumulate at the expense of isothiocyanates. Hydrolysis products of 2-hydroxyalk(en)yl glucosinolates (e.g., progotrin) are unstable as isothiocyanates and undergo rearrangement to oxadolidine-2-thiones. Under acidic conditions in the presence of Fe^{2+} and cysteine, nitriles may accumulate and under neutral

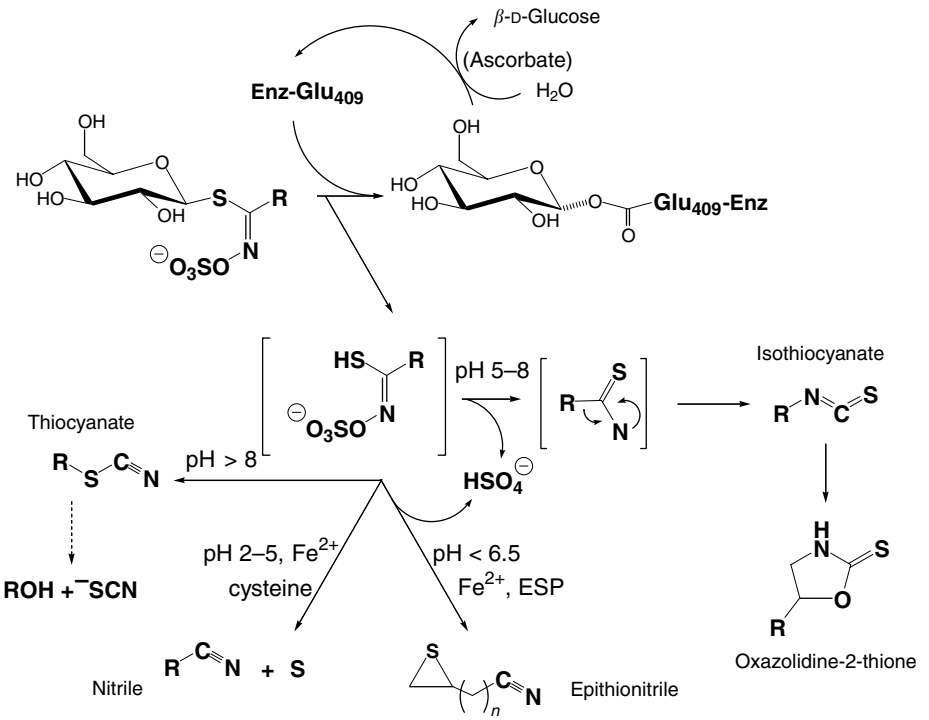


FIGURE 6.50 Reaction mechanism of myrosinase. (Adapted from Burmeister, W.P., et al. (1997). *Structure* 5:663–675; Burmeister, W.P., et al. (2000). *J. Biol. Chem.* 275:39385–39393; and Mithen, R.F., et al. (2000). *J. Sci. Food Agric.* 80:967–984.)

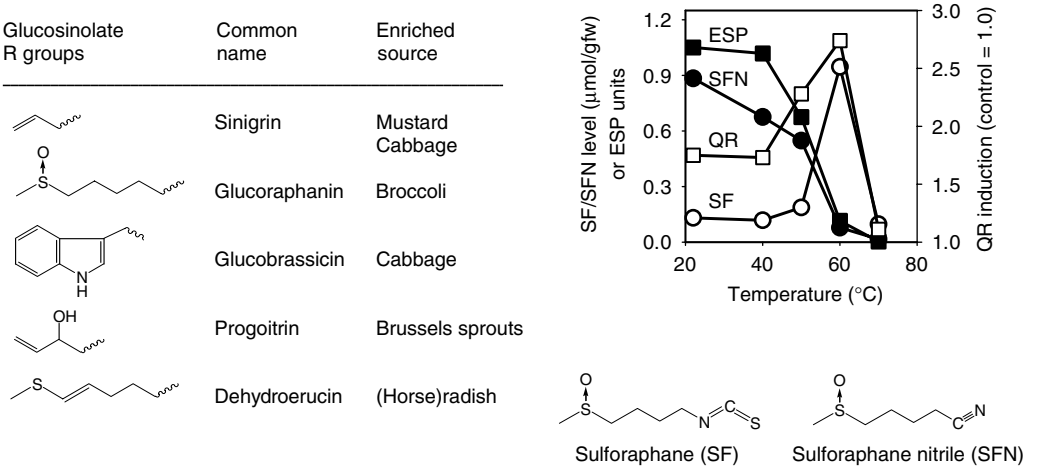


FIGURE 6.51 Representative glucosinolates and control of myrosinase end-product formation. ESP = epithiospecifier protein; QR = quinone reductase, a carcinogen detoxifying enzyme in animal cells. (Adapted from Matusheski, N.V., et al. (2004). *Phytochemistry* 65:1273–1281.)

to slightly alkaline conditions, indole (e.g., glucobrassicin) and benzyl glucosinolates decompose to form the corresponding alcohols and cyanate, while allyl (sinigrin) and methylthio (dehydroerucin) derivatives yield the thiocyanates. Some of these products have antinutritional effects. Cyanates interfere with iodine absorption, the 2-hydroxy-3-butenyl-glucosinolates (progoitrin) are associated

with hypothyroidism, and nitriles may pose toxicity. Despite these concerns, there is a clear association between consumption of *Brassica* spp. vegetables and reduction in cancer risk, and much of this is attributed to glucosinolates and their transformation products.

Sulforaphane, the isothiocyanate derivative formed from glucoraphanin, is believed to be one of the most potent dietary cancer chemopreventive agents derived from broccoli (Chapter 12). However, the sulforaphane nitrile derivative is formed to a greater extent than sulforaphane in broccoli, and the nitrile form has several orders of magnitude less potency in cancer chemopreventive potential than the isothiocyanate form [73]. In addition to its ability to yield epithionitriles, the epithiospecifier protein is associated with nitrile formation from alk(en)yl glucosinolates in other *Brassica* spp., although this remains a subject to debate. In any event, thermal processing was examined as a means to minimize sulforaphane nitrile while maximizing sulforaphane accumulation in broccoli florets (Figure 6.51). A mild thermal treatment of 60°C for 10–20 min retains myrosinase activity and destroys epithiospecifier activity; this preferential deactivation of the latter causes a reversal in ratio of nitrile:isothiocyanate form of sulforaphane from 10:1 to about 1:10. The benefit is to increase the levels of the most potent anticarcinogenic agent in broccoli.

6.5.3.4.2 Alliinases and Related Enzymes [141]

Alliinases (EC 4.4.1.4, alliin alkenyl-sulfenate lyase, or alliin lyase) are the flavor-generating enzymes of members of the Genus *Allium*, including onion, garlic, leek, chive, chemotaxonomically related species such as cabbage, and some mushrooms. The active site architecture and mechanism of alliinase was featured earlier as an example of a pyridoxal-phosphate enzyme, and the reaction involves the β-lysis of nonprotein amino acid derivatives, the *S*-alk(en)yl-L-cysteine sulfoxides (ACSO) (Figure 5.6). The immediate reaction products, the sulfenic acids (R—SOH) spontaneously condense to form thiosulfinates; in onion, much of the 1-propenyl sulfenic acid rearranges to form the propanethial-*S*-oxide, also known as the lachrymatory factor (LF) (Figure 6.52 [114,141]). This reaction is conditional upon rupture of tissues, as the ACSO substrates reside in the cytosol while the enzyme is vacuolar. Most alliinases have similar selectivities with a preference for reaction among ACSO species of descending order: unsaturated (1-propenyl- and 2-propenyl-) > propyl- > methyl-derivatives; reactivity ratios (based on V_{max}/K_M values) of ~10:2:1 [114] represent a middle ground of a wide range of relative selectivity values of alliinases reported in the literature. Consequently, the reaction products and characteristic flavors produced in *Allium* tissues are conferred largely by the relative levels of the various ACSO substrates present (Figure 6.52), rather than by properties of species-specific alliinases.

The enzyme is glycosylated, exists as a limited number of isoforms, and may be oligomeric with monomeric mass typically in the range of 48–54 kDa. One distinction among alliinases is the pH optimum, which is in the range of pH 7–8 for onion, leek, broccoli, and mushroom enzymes, and pH 5.5–6.5 for garlic and related enzymes. However, this difference is of limited practical importance because alliinases are fairly active over the range of pH 4.5–8.5 [60,141], comprise 6 and 10% of the tissue protein, respectively, in onion and garlic, and there is an abundance of activity in disrupted tissues (where pH ranges 5.2–6.0). From 70 to 90% conversion of ACSO by alliinase to organosulfur products occurs in ruptured cells of onion tissue at room temperature within about 1 min, and nearly ~100% conversion occurs in disrupted cells within 1 h [65,101].

Aside from the desirable flavors produced upon tissue disruption, there are several features of alliinase reactions that impact the ability to control food quality. Minced and stored or acidified (pickled) *Allium* tissue preparations may discolor and yield pink/red (in onion) and blue-green (in garlic) hues. The 1-propenyl-S(O)S-R thiosulfinates species are implicated as the major cause of such discoloration [61]. Stored (refrigerated) garlic may accumulate low levels of 1-propenyl-ACSO and the allyl-ACSO contributes to discoloration in minced garlic.

Preserving alliinase activity is important for allowing potentiation of the enzyme reaction at a point of choosing for a tissue preparation. As mentioned earlier, freezing preserves alliinase activity provided thawing is fast enough to prevent excessive denaturation [136]. Cryoprotectants such

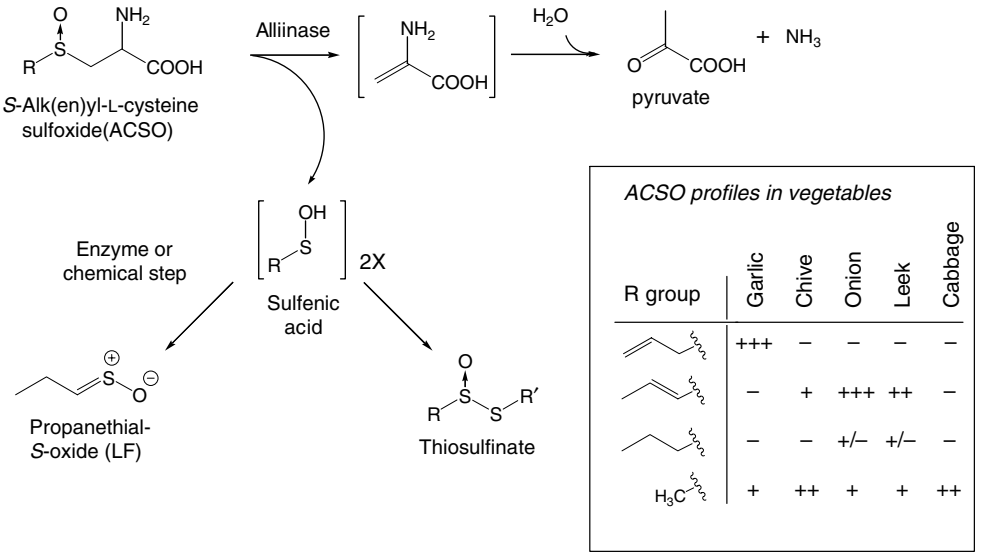


FIGURE 6.52 Reaction pathway of alliinase reactions and profile of substrates in various vegetable tissues. (Compiled from Shen, C. and Parkin, K.L. (2000). *J. Agric. Food Chem.* 48:6254–6260 and Whitaker, J.R., et al. (Eds.) (2003). *Handbook of Food Enzymology*, Marcel Dekker, New York.)

as glycerol and exogenous pyridoxal phosphate cofactor have been routinely added to alliinase preparations to stabilize enzyme activity. Freeze-drying retains about 75% original activity whereas low temperature (55°C) drying retains about 50% original activity [68]. Either of these methods are suitable for preparing *Allium* tissues as dietary supplements where it is desired to have sufficient residual alliinase to generate thiosulfinates *in situ* (in the gut) of humans. This requires the use of enteric-coated capsules or tablets to protect the enzyme from the deactivating effect of gastric acid and enzymes. In contrast, garlic and onion powders prepared for use as spices undergo a more severe thermal treatment and have only ~5% residual alliinase activity.

In *Allium* tissues, some ACSO flavor precursors may exist as γ-glutamyl-ACSO peptides, and these peptide-linked ACSO are not recognized as substrates by alliinase. A transpeptidase (EC 2.3.2.2) catalyzes the transfer of the γ-glutamyl-group from ACSO to another amino acid and liberates free ACSO, which can then be acted upon by alliinase and further potentiate flavor. Sprouting *Allium* bulbs and germinating seeds are particularly rich in transpeptidase activity, and use is made of extracts of such tissues to mobilize a secondary pool of flavor precursors in various *Allium* preparations. Such preparations are most useful in dry form such that reconstitution with aqueous milieu elicits enzyme activities and yield enhanced flavor at a time of choosing.

Cystine lyases (EC 4.4.1.8) also exist in *Allium*, cruciferous, and leguminous plants, as well as in fungi and some bacteria. It is also known as β-cystathionase, although many plant enzymes do not possess both of these activities [141]. Cystine lyases are pyridoxal enzymes that catalyze the β-elimination of cystine to yield thiocysteine (CYS–SSH), and this may give rise to sulfurous flavors. In broccoli, multiple isoforms exist, and they are soluble and have optima at pH 8–9. Depending on source, cystine lyases may also react with ACSO, but alliinases do not react with cystine. A similar pyridoxal enzyme methionine-γ-lyase (EC 4.4.1.11) yields methanethiol (CH_3SH) as a reaction product, and this reaction has been implicated in proper flavor development in some cheeses, likely conferred by starter or adjunct cultures.

6.5.3.4.3 Other Flavor-Related Enzyme Activities

Sweetening through the elevation of maltose in domestically cooked and thermally processed sweet potato products (canned, flakes, puree) is a positive quality trait conferred by endogenous β-amylases

[125]. High-maltose sweet potato lines have greater β-amylase activity with a greater thermal stability. During moderate thermal processing (progressively heated at 70–90°C over 2 h), a faster and greater degree of starch gelatinization in these same lines allows for sustained β-amylase action on starch, leading to up to fivefold greater maltose levels relative to those observed for the moderate- and low-maltose lines.

6.5.4 Enzymes Affecting Textural Quality in Foods

Textural and rheological changes in foods can be evoked by enzymes that act on large and small molecular weight food components. Examples of some textural and rheological modifications have already been described in the context of using exogenous enzymes to liquefy/thin starch, reduce viscosity and cloud in fruit juices, hydrolyze or induce gelation of proteins, modify bread dough viscoelasticity, as well as others. This section will focus on controlling endogenous enzymes that can have desirable or undesirable impact on food quality.

6.5.4.1 Control of Enzymes Modifying Carbohydrate Polymers

Perhaps the most rudimentary yet effective example of controlling endogenous enzyme activity on carbohydrates is the "hot" and "cold" break processes for preparing tomato fruit derivatives. These terms are partly misnomers and a hot break process comprises a rapid heating of tomato tissue to >85–90°C with a clear intent to inactivate endogenous polygalacturonase and pectin methyl esterase activities. This preserves pectin levels, promotes viscosity and consistency, and stabilizes juice cloud. In contrast, a "cold" break process makes use of temperatures <70°C where these enzymes are thermally "activated" and will result in pectin depolymerization with a corresponding loss in viscosity, pectin deesterification leading to loss of cloud stability, reduction in consistency, and separation of serum (liquid) from solids. The cold break process may promote greater flavor quality, perhaps by allowing a greater extent of lipoxygenase/hydroperoxide lyase-mediated flavor generation, but this effect has not been consistently observed. Both cold and hot break processes are used for juice and other fruit products, depending on how those products are to be used (either as end-products or ingredients for others). Tomato pastes are best prepared by hot-break processes to retain consistency and viscosity. High temperature processing is also used for other juicing fruits (orange) to maintain cloud stability as a quality attribute.

Another approach to controlling pectinolytic enzymes for texture control is the application of an intermediate and moderate thermal treatment (referred to as low-temperature blanching) to mitigate softening brought about by subsequent thermal processing of intact (or pieces of) fruit and vegetable products. Treatments in the range of 55–80°C are intended to stimulate pectin methyl esterase action and "firm" tissue by promoting adhesion among cell wall and middle lamella elements. Hydrolysis of pectin methoxy groups creates carboxylate groups that may form Ca^{2+} bridges between neighboring pectin polymers (see egg-box model, Chapter 3). This can enhance textural firmness and prevent disintegration of tissue pieces ("sloughing") during subsequent thermal processing such as retorting.

One of the earliest successes was registered for potatoes [8], where thermal pretreatments of 30–120 min at 60–70°C prior to boiling were effective at preventing excessive softening and almost eliminated sloughing (Figure 6.53 [8]). A minimum temperature of 55–60°C is required to render the tissue "leaky" and permit migration of cations as well as activate pectin methyl esterase, whereas excessive temperatures will deactivate the enzyme before it has sufficient opportunity to act. Thus, potato slices pretreated at 25°C before boiling and those directly boiled, suffer from 80% to 100% disintegration and were more soft after the final boiling step (to simulate retorting) compared to those subjected to a 60–70°C pretreatment followed by boiling. Activation of tissue pectin methyl esterase is indicated by the analysis for increased tissue MeOH and residual enzyme activity over the range of 50–70°C. The same approach has been shown effective for sweet potatoes, snap beans, cucumbers (for pickle preparation), carrots, and pepper and tomato fruit, and in some cases the

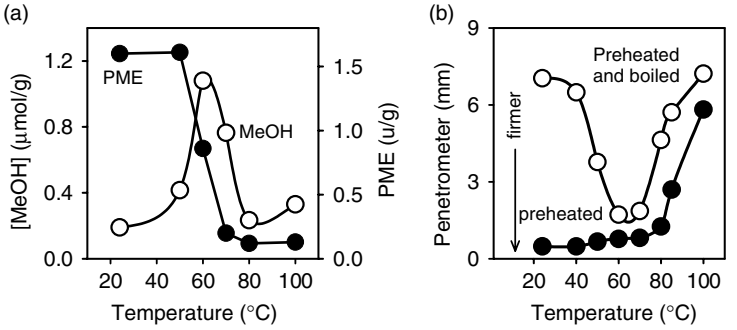


FIGURE 6.53 Thermal potentiation of endogenous pectin methyl esterase (PME) action in potato tuber tissue. (a) Residual MeOH and PME levels in tissue "preheated" for 2 h and (b) tissue firmness after the preheating step (closed symbols) or after both preheating and subsequent boiling steps (open symbols). (Adapted from Bartolome, L.G. and Hoff, J.E. (1972). *J. Agric. Food Chem.* 20:266–270.)

firming effect is enhanced by use of Ca^{2+}-containing brines. An indirect effect of potentiating pectin methyl esterase action is that less substrate will be available for pectin lyases (which recognize methyl-galacturonate residues at the active site, Section 6.3.2.3), conferring less opportunity for β-elimination reactions [132].

6.5.4.2 Control of Enzymes Modifying Proteins

Protein degradation is a major determinant of age tenderization of meat. Cathepsins released from lysosomes and/or calpains are the endogenous muscle proteases that appear to have most impact on tenderization. Other than the temperature and duration of aging, and the early postmortem rate of pH decline, there are few means to influence endogenous proteolysis. One process that has received sustained interest is postmortem electrical stimulation of carcasses, which may confer tenderization by reducing cold shortening, disrupting structural elements of muscle, and stimulating endogenous proteases, in part by Ca^{2+} release into the sarcoplasm [57]. The muscle calpain system, Ca^{2+}-activated CYS-proteases, has been implicated as having a role in early postmortem hydrolysis leading to tenderization. Muscle proteases are just one of several factors that determine tenderness of meats (see Chapter 16).

Endogenous proteases in fish muscle can limit the quality of manufactured gels (surimi products). Proteases in weak gel-forming fish muscle tissue are sensitive to CYS-reactive reagents. An effective way to manage this problem has proven to be the addition of "cystatin"-like protease inhibitors. Such inhibitors are found in bovine plasma, chicken egg white, and potato, and may be added to surimi products to inhibit endogenous protease activity and help maintain gel strength.

Endogenous proteases also occur in milk, the principal one being the plasmin system (derived from the blood). Plasmin (EC 3.4.21.7) is a SER-protease of 81 kDa mass, with reaction optima of pH 7.5–8.0 and 37°C [141]. However, the enzyme is stable over a broad pH range of 4–9 and exhibits 20% maximal activity at 5°C. Plasmin survives pasteurization, owing to its multiple disulfide bonds, and it also retains activity after ultra-pasteurization. Plasminogen (the zymogen form) is dominant in milk and is transformed by activators (including another SER-protease) to yield the active plasmin. Both plasmin(ogen) and its activators are associated with casein micelles. Inhibitors of plasmin and plasmin activators reside in the serum phase and prevent spontaneous activation and proteolysis in freshly drawn and pasteurized milk. During cheese-making, plasmin remains with the casein micelles, and it contributes to proteolysis of particularly α_{s2}- and β-caseins in cheese during aging. In cheese made from ultrafiltered milk, plasmin may be less active because of greater retention of the serum solids (the source of plasmin inhibitors) in the resulting curd. Because of the heat resistance of plasmin, it is a major contributor to proteolysis in cheese subjected to high cook temperatures, which

may inactivate plasmin inhibitors. Owing to its heat resistance, plasmin has also been implicated in having a role in gelation of ultra-high pasteurized milk and creamers.

6.5.4.3 Amelioration of Texture Defects Using Small Molecules to Control Enzymes

In dates, a defect known as "sugar wall" occurs when the sucrose:reducing sugar ratio is sufficiently high (2:1) to cause sucrose crystallization throughout the fruit and yield a sandy, hard texture [118]. Natural and dry dates of premium grade have sugar ratios in the range of 1.1–1.6:1 by comparison, and dates prone to sugar wall tend to have low endogenous invertase levels. To reduce the incidence of this defect, sugar wall dates were subjected to a vacuum-infusion treatment containing 0.01–0.10% commercial invertase, with a sample comprising the control treatment sprayed with an equivalent amount of water alone. After the treatment, which resulted in increased moisture contents to 20–22% (and an increase in a_w), the dates were sealed and stored for 60 days at ~27°C. As expected, the enzyme-infused dates exhibited an "inversion" of 54–76% of the sucrose, lowering the sucrose:reducing sugar ratio to 0.22–0.44:1. Surprisingly, even in the water-infused dates, sucrose inversion amounted to 53% with a reduction in sucrose:reducing sugar ratio to 0.56:1. The sugar wall defect was not evident after the 60-day treatment period for enzyme- and water-infused samples. This illustrates how facile it can sometimes be to potentiate endogenous activity, in this case, by simple water addition. At the end of the 60-day period, all treated and control dates were dried to the 16–18% original moisture content and observed for another month. All water-infused samples returned to the sugar wall defect, while the enzyme-infused samples showed 0–10% incidence of the defect returning, inversely related to the enzyme dose. Thus, permanent elimination of the defect requires treatment with exogenous invertase.

The last example of control of enzyme action in foods deals with TMAO demethylase (EC 4.1.2.32), which causes a reaction in muscle, particularly for fish of the Gadoid (cod) family:

$$(CH_3)_3N{\rightarrow}O \longrightarrow (CH_3)_2NH + HCHO \tag{6.59}$$

The formaldehyde (HCHO) produced causes protein cross-linking, rendering the muscle tissue tough and fibrous when stored frozen as fillets or in blocks/portions. Tissue disruption, by freezing or simple mincing, causes the enzyme reaction to occur by decompartmentation (TMAO can be >100 mM in muscle). TMAO demethylase is not widely distributed but occurs in some bacteria. In fish muscle and organ tissues, it appears to be membrane associated, but can be solubilized. Two "cofactor" or cosubstrate systems were shown to mediate reactivity for the isolated membrane enzyme [87]. The cyanide-sensitive one requires NAD(P)H and FMN, and functions only anaerobically. The other is cyanide insensitive, involves Fe^{2+}, ascorbate, and/or cysteine, and functions independent of oxygen tension but is only 20% as stimulatory as the NAD(P)H/FMN system.

It is commercially important to prevent this reaction in frozen fish blocks (~7 kg of rectangular dimension), which are processed later into portions, and "aging" on ice prior to freezing was considered as a practical approach [100]. Rates of TMAO formation increase progressively toward the more anaerobic interior of blocks prepared from fresh (0 days aged) fish fillets (Table 6.13 [100]). However, this depth effect quickly diminished after only 1 day of aging and was completely lacking after 10 days of aging. Thus, anaerobic enzyme reaction capacity was lost rapidly during aging, and it is likely that the cofactors, NAD(P)H and FMN, required for this purpose decayed in aging muscle and could not be replenished [90]. This is also reinforced by the effect of KCN, a hemoprotein-inhibitor, which reduced the rate of TMAO formation in unaged fish to a normalized rate at all depths into the block. KCN-inhibition of the anaerobic enzyme system leaves only the O_2-independent enzyme activity to function. This study indicates that the anaerobic cofactor system is most active in fish muscle and the major "cofactor" system supporting enzyme activity can be depleted simply by allowing the fish to age on ice before freezing. This example illustrates control

TABLE 6.13
Control of TMAO Demethylase Activity in Frozen Fish Muscle

Position in Block (Depth in mm)	Initial Reaction Rate (μmol HCHO/100 g per day) after Aging on Ice for			
	0 days	1 day	10 days	0 day + KCN
2	10	7.0	2.4	3.9
6	20	8.5	2.4	3.5
10	24	11	2.3	2.1
18	25	12	2.1	4.0

Note: KCN was used at 2 mM. Blocks (160 × 100 × 25 mm) were stored at −10°C.

Source: Reece, P. (1983). *J. Sci. Food Agric.* 34:1108–1112.

of enzyme action in foods by strategies targeting cofactor, activators, or substrate disposition. An alternative approach to managing substrate/cofactor levels in fish susceptible to this textural problem was based on a Maine fishermen's suggestion to soak/freeze the fillets in seawater [66]. This allows for a proportion of the low molecular weight constituents, including substrate and cofactors, to be osmotically leached out of the muscle. Subsequent freezing led to ∼80% reduction in rate and extent of HCHO formation and less textural deterioration.

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Part II

Minor Food Components

7 Vitamins

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CONTENTS

7.1 INTRODUCTION

7.1.1 Objectives

Since the discovery of the basic vitamins and their many forms, a wealth of information has been generated and published on their retention in foods during postharvest handling, commercial processing, distribution, storage, and preparation, and many reviews have been written on this topic. A good summary of older findings regarding this topic is *Nutritional Evaluation of Food Processing* [62,63,77], to which the reader is encouraged to refer. There is a need for an in depth review of more recent literature.

The major objective of this chapter is to discuss and critically review the chemistry of the individual vitamins and our understanding of the chemical and physical factors that influence vitamin retention and bioavailability in foods. A secondary objective is to indicate gaps in our understanding and to point out factors that affect the quality of data with respect to our understanding of vitamin stability. It should be noted that there is an unfortunate state of inconsistency of nomenclature in the vitamin literature, with many obsolete terms still being used. Throughout this chapter, terminology recommended by the International Union of Pure and Applied Chemistry (IUPAC) and the American Society for Nutritional Sciences [1] will be used.

7.1.2 Summary of Vitamin Stability

The vitamins comprise a diverse group of organic compounds that are nutritionally essential micronutrients. Vitamins function *in vivo* in several ways, including: (1) as coenzymes or their precursors (niacin, thiamin, riboflavin, biotin, pantothenic acid, vitamin B_6, vitamin B_{12}, and folate); (2) as components of the antioxidative defense system (ascorbic acid (AA), certain carotenoids, and vitamin E); (3) as factors involved in genetic regulation (vitamins A, D, and potentially several others); and (4) in specialized functions such as vitamin A in vision, ascorbate in various hydroxylation reactions, and vitamin K in specific carboxylation reactions.

Vitamins are quantitatively minor constituents of foods. From the viewpoint of food chemistry, we are mainly interested in maximizing vitamin retention by minimizing aqueous extraction (leaching) and chemical changes such as oxidation and reaction with other food constituents. In addition, several of the vitamins influence the chemical nature of food, by functioning as reducing agents, radical scavengers, reactants in browning reactions, and as flavor precursors. Although much is known about the stability and properties of vitamins, our knowledge of their behavior in the complex milieu of food is limited. Many published studies have, sometimes by necessity, involved the use of chemically defined model systems (or even just buffer solutions) to simplify the investigation of vitamin stability. Results of such studies should be interpreted with caution because, in many cases, the degree to which these model systems simulate complex food systems is not known. Although these studies have provided important insight into chemical variables affecting retention, they are sometimes of limited value for predicting the behavior of vitamins in complex food systems. This is so because complex foods often differ markedly from model systems in terms of physical and compositional variables including water activity, ionic strength, pH, enzymatic and trace metallic catalysts, and other reactants (protein, reducing sugars, free radicals, active oxygen species, etc.). Throughout this chapter, emphasis will be placed on the behavior of vitamins under conditions relevant to actual foods.

Most of the vitamins exist as groups of structurally related compounds exhibiting similar nutritional function. Many attempts have been made to summarize the stability of the vitamins, such as

TABLE 7.1
Summary of Vitamin Stability[a]

Nutrient	Neutral	Acid	Alkaline	Air or Oxygen	Light	Heat	Maximum Cooking Loss (%)
Vitamin A	S	U	S	U	U	U	40
Ascorbic acid	U	S	U	U	U	U	100
Biotin	S	S	S	S	S	U	60
Carotenes	S	U	S	U	U	U	30
Choline	S	S	S	U	S	S	5
Vitamin B_{12}	S	S	S	U	U	S	10
Vitamin D	S	S	U	U	U	U	40
Folate	U	U	U	U	U	U	100
Vitamin K	S	U	U	S	U	S	5
Niacin	S	S	S	S	S	S	75
Pantothenic acid	S	U	U	S	S	U	50
Vitamin B_6	S	S	S	S	U	U	40
Riboflavin	S	S	U	S	U	U	75
Thiamin	U	S	U	U	S	U	80
Tocopherols	S	S	S	U	U	U	55

Note: Caution: these conclusions are oversimplifications and may not accurately represent stability under all circumstances.

[a] S, stable (no important destruction); U, unstable (significant destruction).

Source: Adapted from Harris, R. S. (1971). General discussion on the stability of nutrients, in *Nutritional Evaluation of Food Processing* (R. S. Harris and H. von Loesecke, eds.), AVI Publishing Co., Westport, CT, pp. 1–4. With modifications.

that shown in Table 7.1 [61]. The major limitation of such generalization is the marked variation in stability that can exist among the various forms of each vitamin. For example, tetrahydrofolic acid and folic acid are two folates that exhibit nearly identical nutritional properties. However, as described later, tetrahydrofolic acid (a naturally occurring form) is extremely susceptible to oxidative degradation, while folic acid (a synthetic form used in food fortification) is very stable. Thus, attempts to generalize or summarize the properties of vitamins are at best imprecise and at worst highly misleading.

7.1.3 Toxicity of Vitamins

In addition to the nutritional role of vitamins, it is important to recognize their potential toxicity. Vitamins A, D, and B_6 are of particular concern in this respect. Episodes of vitamin toxicity are nearly always associated with overzealous consumption of nutritional supplements. Toxic potential also exists from inadvertent excessive fortification, as has occurred in an incident with vitamin D-fortified milk. This illustrates the need for continued monitoring by regulatory and public health agencies. Instances of intoxication from vitamins occurring endogenously in food are exceedingly rare.

7.1.4 Sources of Vitamins

Although vitamins are consumed in the form of supplements by a growing fraction of the population, in many cases the food supply generally represents the major and most critically important source of vitamin intake. Foods, in their widely disparate forms, provide vitamins that occur naturally in plant, animal, and microbial sources as well as those added in fortification. In addition, certain dietetic and

medical foods, enteric formulas, and intravenous solutions are formulated so that the entire vitamin requirements of the individual are supplied from these sources.

Regardless of whether the vitamins are naturally occurring or added, the potential exists for losses by chemical or physical (leaching or other separations) means. Losses of vitamins are, to some degree, inevitable in the manufacturing, distribution, marketing, home storage and preparation of processed foods, and losses of vitamin can also occur during the postharvest handling and distribution of fruits and vegetables and during the postslaughter handling and distribution of meat products. Since the modern food supply is increasingly dependent on processed and industrially formulated foods, the nutritional adequacy of the food supply depends, in large measure, on our understanding of how vitamins are lost and on our ability to control these losses.

Considerable information is available concerning the stability of vitamins in foods. However, our ability to use such information is frequently limited by a poor understanding of reaction mechanisms, kinetics, and thermodynamics under various conditions. Thus, it is frequently difficult on the basis of our present knowledge to predict the extent to which given processing, storage, or handling conditions will influence the retention of many vitamins. Without sufficient knowledge of reaction kinetics and thermodynamics, it is difficult to select conditions and methods of food processing, storage, and handling to optimize vitamin retention. Thus, there is a great need for more thorough characterization of the basic chemistry of vitamin degradation as it occurs in complex food systems.

7.2 ADDITION OF NUTRIENTS TO FOODS

Throughout the early twentieth century, nutrient deficiency represented a major public health problem in the United States. Pellagra was endemic in much of rural South, while deficiencies of riboflavin, niacin, iron, and calcium were widespread. The development of legally defined standards of identity under the authorization of the 1938 Food, Drug, and Cosmetic Act provided for the direct addition of several nutrients to foods, especially certain dairy and cereal-grain products. Although technological and historical aspects of fortification are beyond the scope of this chapter, the reader is referred to *Nutrient Additions to Food, Nutritional, Technological, and Regulatory Aspects* [7] for a comprehensive discussion of this topic. The nearly complete eradication of overt vitamin-deficiency disease provides evidence of the exceptional effectiveness of fortification programs and the general improvement in the nutritional quality of the U.S. food supply.

Definitions of terms associated with the addition of nutrients to foods include:

1. *Restoration*: Addition to restore the original concentration of key nutrients.
2. *Fortification*: Addition of nutrients in amounts significant enough to render the food a good to superior source of the added nutrients. This may include addition of nutrients not normally associated with the food or addition to levels above that present in the unprocessed food.
3. *Enrichment*: Addition of specific amounts of selected nutrients in accordance with a standard of identity as defined by the U.S. Food and Drug Administration (FDA).
4. *Nutrification*: This is a generic term intended to encompass any addition of nutrients to food.

The addition of vitamins and other nutrients to food, while clearly beneficial in current practice, also carries with it the potential for abuse and, thus, risk to consumers. For these reasons, important guidelines have been developed, which convey a reasonable and prudent approach. These U.S. FDA guidelines [21 CFR Section 104.20(g)] state that the nutrient added to a food should be:

1. Stable under customary conditions of storage, distribution and use;
2. Physiologically available from the food;

3. Present at a level where there is assurance that there will not be excessive intake;
4. Suitable for its intended purpose and in compliance with provisions (i.e., regulations) governing safety.

Further, it is stated in these guidelines that "the FDA does not encourage the indiscriminant addition of nutrients to foods." Similar recommendations have been developed and endorsed jointly by the Council on Foods and Nutrition of the American Medical Association (AMA), the Institute of Food Technologists (IFT), and the Food and Nutrition Board (FNB) of the National Academy of Sciences-National Research Council [4].

Additionally, AMA-IFT-FNB guidelines recommend that the following prerequisites be met to justify fortification: (1) the intake of the particular nutrient is inadequate for a substantial portion of the population; (2) the food (or category) is consumed by most individuals in the target population; (3) there is reasonable assurance that excessive intake will not occur; and (4) the cost is reasonable for the intended population. The joint statement also included the following endorsement of enrichment programs. Specifically the following practices in the United States continue to be endorsed:

> The enrichment of flour, bread, degerminated and white rice (with thiamin, riboflavin, niacin, and iron); the retention or restoration of thiamin, riboflavin, niacin, and iron in processed food cereals; the addition of vitamin D to milk, fluid skimmed milk, and nonfat dry milk, the addition of vitamin A to margarine, fluid skim milk, and nonfat dry milk, and the addition of iodine to table salt. The protective action of fluoride against dental caries is recognized and the standardized addition of fluoride is endorsed in areas in which the water supply has a low fluoride content.

In addition, as of January 1, 1998, the inclusion of folic acid is required in enriched cereal-grain products (i.e., those with standards of identity, including most wheat flours, rice, corn meals, breads, and pastas). This has proven to be a viable approach to providing supplemental folic acid for the purpose of reducing the risk of certain birth defects (spina bifida and anencephaly), and it has improved the folate nutritional status of the population. The level of addition of folic acid was chosen to minimize the risk of excessive intake (>1 mg folic acid/d) to reduce the risk of masking the diagnosis of vitamin B_{12} deficiency. However, recent evidence of overfortification with folic acid illustrates the need for careful monitoring of any fortification program.

The stability of vitamins in fortified and enriched foods has been thoroughly evaluated. As shown in Table 7.2, the stability of added vitamins in enriched cereal grain products under conditions of accelerated shelf life testing is excellent [3,21]. Similar results have been reported with fortified breakfast cereals (Table 7.3). This excellent retention is due, in part, to the stability of the chemical forms of these vitamins used, as well as the favorable environment with respect to water activity and temperature. The stability of vitamins A and D in fortified milk products also has been shown to be satisfactory.

7.3 DIETARY RECOMMENDATIONS

To assess the impact of food composition and intake patterns on the nutritional status of individuals and populations, and to determine the nutritional effects of particular food processing and handling practices, a nutritional reference standard is essential. In the United States, the Recommended Dietary Allowances (RDA) have been developed for these purposes. The RDA values have been defined by the Committee on Dietary Allowances of the Institute of Medicine's FNB as "the average daily dietary intake level that is sufficient to meet the nutrient requirement of nearly all (97–98%) healthy individuals in a particular stage of life and gender group" [70]. To the extent possible, the RDA values are formulated to include allowances for variability within the population with respect to nutrient requirements as well as the potential for incomplete bioavailability of nutrients. However, limitations in our current knowledge of the bioavailability of vitamins in foods render

TABLE 7.2
Stability of Vitamins Added to Cereal Grain Products

			Storage Time (Months @23°C)		
Vitamin	**Claim**	**Found**	**2**	**4**	**6**
In 1 lb of white flour					
Vitamin A (IU)	7500	8200	8200	8020	7950
Vitamin E (IU)[a]	15.0	15.9	15.9	15.9	15.9
Pyridoxine (mg)	2.0	2.3	2.2	2.3	2.2
Folate (mg)	0.30	0.37	0.30	0.35	0.3
Thiamin (mg)	2.9	3.4		3.4	
In 1 lb of yellow corn meal					
Vitamin A (IU)		7500	7500		6800
Vitamin E (IU)[a]		15.8	15.8	15.9	
Pyridoxine (mg)		2.8	2.8		2.8
Folate (mg)		0.30	0.30		0.29
Thiamin (mg)		3.5			3.6
	After baking		5 days storage (23°C)		
In 740 g of bread					
Vitamin A (IU)	7500	8280	8300		
Vitamin E (IU)[a]	15	16.4	16.7		
Pyridoxine (mg)	2	2.4	2.5		
Folate (mg)	0.3	0.34	0.36		

[a] Vitamin E is expressed as DL-α-tocopherol acetate.

Source: Cort, W. M., et al. (1976). *Food Technol.* 30:52–62.

TABLE 7.3
Stability of Vitamins Added to Breakfast Cereal Products

		Storage Time	
Vitamin Content (Per g of Product)	**Initial Value**	**Three Months (40°C)**	**Six Months (23°C)**
Vitamin A (IU)	193	168	195
Ascorbic acid (mg)	2.6	2.4	2.5
Thiamin (mg)	0.060	0.060	0.064
Riboflavin (mg)	0.071	0.074	0.67
Niacin (mg)	0.92	0.85	0.88
Vitamin D	17.0	15.5	16.6
Vitamin E (IU)	0.49	0.49	0.46
Pyridoxine (mg)	0.085	0.088	0.081
Folate (mg)	0.018	0.014	0.018
Vitamin B_{12} (μg)	0.22	0.21	0.21
Pantothenic acid (mg)	0.42	0.39	0.39

Source: Anderson, R. H., et al. (1976). *Food Technol.* 30:110–114.

such allowances somewhat uncertain. Many other countries and several international organizations such as the FAO/WHO have developed reference values similar to the RDAs, and these sometimes differ quantitatively because of differences in scientific judgment or philosophy.

For food labeling to be meaningful, the concentration of micronutrients is best expressed relative to reference values. In the United States, nutrition labeling data for micronutrients has been traditionally expressed as a percentage of a "U.S. RDA" value, a practice that was originated at the onset of nutrition labeling in the early 1970s. The U.S. RDAs currently used for nutrition labeling were derived from the 1968 RDA values and differ somewhat from the current RDA values reported by the FNB (Table 7.4) [70–72]. These differences, although not readily evident to the consumer, should be recognized and understood. Federal regulations permit modification of U.S. RDAs by the FDA "from time to time as more information on human nutrition becomes available" [21 CFR §101.9(c)(7)(b)(ii)], although no changes have yet been implemented. Under the revised labeling regulation implemented by the FDA in 1994, the U.S. RDA term has been replaced by the "Reference Daily Intake (RDI)," which is currently equivalent to the previous U.S. RDAs. In the current nutrition labeling format, vitamin content is expressed as a percentage of the RDI and is listed on labels as "% Daily Value."

7.4 ANALYTICAL METHODS AND SOURCES OF DATA

The major sources of information regarding the content of vitamins in U.S. foods are the U.S. Department of Agriculture's National Nutrient Data Bank and the Agricultural Handbook No. 8 series. An important limitation of these data and most other databases is the uncertain adequacy of the analytical methods used. It is frequently unclear how the data were obtained, what methods were used, and whether the results were based on truly representative samples. Issues regarding the merits of information in nutrient databases have been discussed in several reviews [8,68].

The adequacy of analytical methods is a serious problem with respect to many vitamins. While current analytical methods are generally acceptable for some vitamins (e.g., ascorbic acid, thiamin, riboflavin, niacin, vitamin B_6, vitamin A, and vitamin E), they are less adequate for others (e.g., folate, pantothenic acid, biotin, carotenoids, vitamin B_{12}, vitamin D, and vitamin K). Factors that limit the suitability of analytical methods may involve a lack of specificity of traditional chemical methods, interferences in microbiological assays, incomplete extraction of the analyte(s) from the food matrix, and incomplete measurement of complexed forms of a vitamin. Improvement of analytical data for vitamins will require additional support for methods development research, improved training of analysts, development of quality control protocols (i.e., validation and standardization of procedures), and development of standard reference materials for vitamin analysis. The strengths and limitations of analytical methods for each vitamin will be briefly addressed in this chapter.

7.5 BIOAVAILABILITY OF VITAMINS

The term bioavailability refers to the degree to which an ingested nutrient undergoes intestinal absorption and metabolic function or utilization within the body. In the broad sense, bioavailability involves both absorption and utilization of the nutrient *as consumed*. This concept does not refer to losses that may occur before consumption. For a complete description of the nutritional adequacy of a food, three factors must be known: (1) the concentration of the vitamin *at the time of consumption*; (2) the identity of various chemical species of the vitamin present; and (3) the bioavailability of these forms of the vitamin *as they exist in the meal consumed*.

Factors that influence the bioavailability of vitamins include (1) composition of the diet, which could influence intestinal transit time, viscosity, emulsion characteristics, and pH; (2) form of the vitamin (forms may differ in rate or extent of absorption, stability in the stomach and intestine prior to digestion, ease of conversion to metabolically active or coenzymic form, or metabolic functionality); (3) interactions between a vitamin and components of the diet (e.g., proteins, starches, dietary fiber,

TABLE 7.4
Comparison of Recommended Dietary Allowances for Vitamins and "Reference Daily Intake" (RDI) Currently Used in Nutritional Labeling in the United States

Category	Age (yr) or Condition	Vitamin A (μg R.A.E.)[a]	Vitamin D (μg)	Vitamin E (mg as α-toc.)	Vitamin K (μg)	Vitamin C (mg)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg NE)	Vitamin B_6 (mg)	Folate (μg DFE)	Vitamin B_{12} (μg)	Pantothenic Acid (mg)	Biotin (μg)	Choline (mg)
Infants	0.0–0.5	400	5	4	2.0	40	0.2	0.3	2	0.1	65	0.4	1.7	5	125
	0.5–1.0	500	5	5	2.5	50	0.3	0.4	4	0.3	80	0.5	1.8	6	150
Children	1–3	300	5	6	30	15	0.5	0.5	6	0.5	150	0.9	2	8	200
	4–8	400	5	7	55	25	0.6	0.6	8	0.6	200	1.2	3	12	200
Males	9–13	600	5	11	60	45	0.9	0.9	12	1.0	300	1.8	4	20	375
	14–18	900	5	15	75	75	1.2	1.3	16	1.3	400	2.4	5	25	550
	19–30	900	5	15	120	90	1.2	1.3	16	1.3	400	2.4	5	30	550
	31–50	900	5	15	120	90	1.2	1.3	16	1.3	400	2.4	5	30	550
	51–70	9005	10	15	120	90	1.2	1.3	16	1.7	400	2.4	5	30	550
	>70	900	10	15	120	90	1.2	1.3	16	1.0	400	2.4	5	30	550
Females	9–13	600	5	11	60	90	0.9	0.9	12	1.2	300	1.8	4	20	375
	14–18	700	5	15	75	90	1.0	1.0	14	1.3	400	2.4	5	25	400
	19–30	700	5	15	90	90	1.1	1.1	14	1.3	400	2.4	5	30	425
	31–50	700	5	15	90	90	1.1	1.1	14	1.3	400	2.4	5	30	425
	51–70	700	10	15	90	90	1.1	1.1	14	1.5	400	2.4	5	30	425
	>70	700	10	15	90	90	1.1	1.1	14	1.5	400	2.4	5	30	425
Pregnant	<18	750	5	15	75	80	1.4	1.4	18	1.9	600	2.6	6	30	450
	19–30	770	5	15	90	85	1.4	1.4	18	1.9	600	2.6	6	30	450
	31–50	770	5	15	90	85	1.4	1.4	18	1.9	600	2.6	6	30	450
Lactating	<18	1200	5	19	75	115	1.4	1.6	17	2.0	500	2.8	7	35	550
	19–30	1300	5	19	90	120	1.4	1.6	17	2.0	500	2.8	7	35	550
	31–50	1300	5	19	90	120	1.4	1.6	17	2.0	500	2.8	7	35	550
RDI[b] (used in food labeling)		1000 (5000 IU)	10 (400 IU)	20 (30 IU)	no RDI	60	1.5	1.7	20	2.0	400	6.0	no RDI	no RDI	no RDI

[a] Units (per day): RE, retinol equivalent (1 μg RAE = 1 μg retinol or 12 μg β-carotene, 24 μg a-carotene, 24 μg cryptoxanthin); Vitamin E as α-tocopherol, α-tocopherol equivalent; NE, niacin equivalent (1 mg NE = 1 mg niacin or 60 mg tryptophan); DFE, dietary folate equivalent (μg DFE = μg naturally occurring food folate + 1.7 × μg synthetic folic acid).
[b] Reference Daily Intake is the reference unit used in U.S. nutrition labeling of foods, formerly termed the U.S. RDA.

Source: From Food and Nutrition Board [70–72].

and lipids) that interfere with intestinal absorption of the vitamin. Although our understanding of the relative bioavailability of the various species of each vitamin is rapidly improving, the complex influences of food composition on vitamin bioavailability remain poorly understood. In addition, the effects of processing and storage on vitamin bioavailability have been only partially determined.

Bioavailability is generally considered in the development of dietary recommendations (e.g., RDA values), but this involves only the use of estimated mean bioavailability values. At the present time, our knowledge is too fragmentary to permit vitamin bioavailability data to be included in food composition tables. However, even if our understanding of the bioavailability of vitamins in individual foods were much more complete, such data regarding *individual foods* may be of little use. A far greater need is for a better understanding of vitamin bioavailability in the *diet as a whole* (including interactive effects of individual foods) and the sources of variation in this respect among individual people.

7.6 GENERAL CAUSES OF VARIATION/LOSSES OF VITAMINS IN FOOD

Beginning at the time of harvesting, all foods inevitably undergo some loss of vitamins. The nutritional significance of partial loss of vitamins depends on the nutritional status of the individual (or population) for the vitamin of interest, the importance of the particular food as a source of that vitamin, and the bioavailability of the vitamin. Most processing, storage, and handling methods are intended to minimize vitamin losses. The following is a summary of the various factors responsible for variation in the vitamin content of foods.

7.6.1 Inherent Variation in Vitamin Content

The concentration of vitamins in fruits and vegetables often varies with the genetic characteristics of the cultivar, stage of maturity, site of growth, and climate. During maturation of fruits and vegetables, vitamin concentration is determined by the rates of synthesis and degradation. Information on the time course of vitamin concentration in most fruits and vegetables is not available except for ascorbic acid and β-carotene in a few products. In the example shown in Table 7.5 [95], the maximum concentration of ascorbic acid in tomatoes occurred before full maturity. A similar phenomenon has been seen in recent studies of folate in tomatoes, with a 35% reduction observed during ripening.

TABLE 7.5
Influence of Degree of Maturity on Ascorbic Acid Content of Tomatoes

Weeks from Anthesis	Mean Fruit Weight (g)	Color	Ascorbic Acid (mg/100 g)
2	33.4	Green	10.7
3	57.2	Green	7.6
4	102	Green–yellow	10.9
5	146	Yellow–red	20.7
6	160	Red	14.6
7	168	Red	10.1

Source: Malewski, W. and P. Markakis. (1971). A research note. Ascorbic acid content of developing tomato fruit. *J. Food Sci.* 36:537.

A study of carrots showed that carotenoid concentration varied markedly with variety but was not influenced significantly by stage of maturity.

Little is known about developmental changes in vitamin content of cereal grains and legumes. In contrast to fruits and vegetables, cereal grains and legumes are harvested at a fairly uniform stage of maturity.

Agricultural practices and environmental conditions undoubtedly influence the content of vitamins in plant-derived foods, but few data are available on this subject. Klein and Perry [83] determined the content of ascorbic acid and vitamin A activity (from carotenoids) in selected fruits and vegetables sampled from six different locations across the United States. In their study, wide variation was found among sampling sites, possibly as a result of geographic/climatic effects, varietal differences, and effects of local agricultural practices. Variations and interactions among agricultural practices, including type and amount of fertilizer and irrigation regimen, environment, and genetics would certainly influence vitamin content of plant-derived foods, but these relationships would be very difficult to characterize in a systematic fashion. It is likely that various plants will be genetically engineered in the near future to produce increased amounts of certain vitamins (e.g., folate) or vitamin-active compounds (e.g., β-carotene) to achieve "biofortification" [28].

The vitamin content of animal products is governed both by biological control mechanisms and the diet of the animal. In the case of many B-vitamins, the concentration of the vitamin in tissues is limited by the capacity of the tissues to take up the vitamin from the blood and to convert it to the coenzymic form(s). A nutritionally inadequate animal diet can yield reduced tissue concentrations of water-soluble and fat-soluble vitamin(s). In contrast to the situation with water-soluble vitamins, dietary supplementation with fat-soluble vitamins can more readily increase tissue concentrations. This has been examined as a means of increasing the vitamin E concentration of certain animal products to improve oxidative stability and color retention.

7.6.2 Postharvest Changes in Vitamin Content of Foods

Fruits, vegetables, and animal tissues often retain enzymatic activities that contribute to postharvest changes in the vitamin content of foods. The release of oxidative and hydrolytic enzymes, as a result of deterioration of cellular integrity and compartmentation, can cause changes in the distribution of chemical forms and activity of vitamins. For example, dephosphorylation of vitamin B_6, thiamin or flavin coenzymes, deglycosylation of vitamin B_6 glucosides, and the deconjugation of polyglutamyl folates can cause differences between postharvest distributions and those occurring naturally in the plant or animal before harvest or slaughter. The extent of such changes will depend on physical damage encountered during handling, possible temperature abuse, and the length of time between harvest and processing. Such changes will have little influence on the net concentration of a vitamin but may influence its bioavailability. In contrast, indirect oxidative changes caused by the action of lipoxygenases can reduce the concentration of many vitamins, while AA oxidase can specifically reduce the concentration of AA.

Postharvest changes in vitamin concentration are inevitable but can be minimized when proper postharvest handling procedures are followed. The mishandling of plant products through prolonged holding or shipment at ambient temperatures can contribute to major losses of labile vitamins. Since plant tissues are metabolically active, changes in total concentration as well as distribution of chemical forms of certain vitamins can occur depending on the storage conditions. Postharvest losses of vitamins in meat products are usually minimal under typical conditions of refrigerated storage.

7.6.3 Preliminary Treatments: Trimming, Washing, and Milling

The peeling and trimming of fruits and vegetables can cause losses of vitamins to the extent that they are concentrated in the discarded stem, skin, or peel fractions. Although this can be a source of

significant loss relative to the intact fruit or vegetable, in most cases this must be considered to be an inevitable loss regardless of whether it occurs in industrial processing or home preparation.

Alkaline treatments to enhance peeling can cause losses of labile vitamins such as folate, AA, and thiamin at the surface of the product. However, losses of this kind tend to be small compared to the total vitamin content of the product.

Any exposure of cut or otherwise damaged tissues of plant or animal products to water or aqueous solutions causes the loss of water-soluble vitamins by extraction (leaching). This can occur during washing, transportation via flumes, and during exposure to brines during cooking. The extent of such losses depends on factors that influence the diffusion and solubility of the vitamin, including: pH (can effect solubility and dissociation of vitamins from binding sites within the tissue), ionic strength of the extractant, temperature, the volume ratio of food to aqueous solution, and the surface-to-volume ratio of the food particles. After extraction, the destruction of the vitamin depends on the concentration of dissolved oxygen, ionic strength, concentration and type of catalytic trace metals, and the presence of other destructive (e.g., chlorine) or protective (e.g., certain reducing agents) components in the medium.

The milling of cereal grains involves grinding and fractionation to remove the bran (seed coat) and germ. Because many vitamins are concentrated in the germ and bran, major losses of vitamins can occur during their removal (Figure 7.1) [102]. Such losses, as well as the prevalence of vitamin deficiency diseases, provided the rationale for initiating the enrichment of cereal grain products with several added nutrients (riboflavin, niacin, thiamin, iron, and calcium) and, more recently, folic acid. The beneficial impact of this enrichment program on public health has been enormous.

7.6.4 Effects of Blanching and Thermal Processing

Blanching, a mild heat treatment, is an essential step in the processing of fruits and vegetables. The primary purposes are to inactivate potentially deleterious enzymes, reduce microbial loads, and

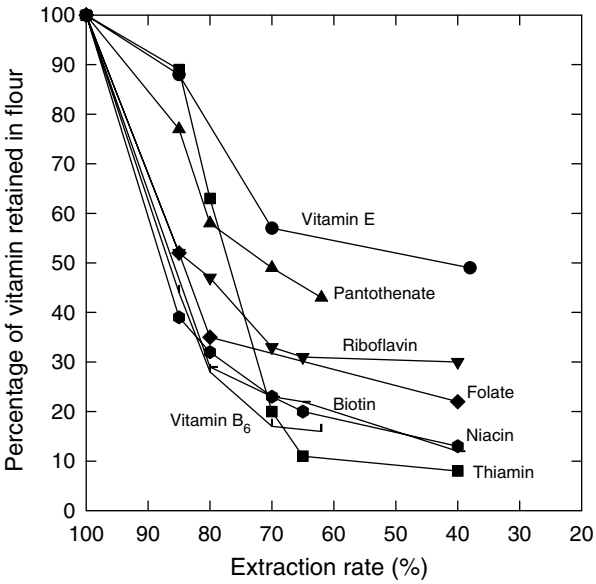


FIGURE 7.1 Retention of selected nutrients as a function of degree of refining in production of wheat flour. Extraction rate is the term used in milling referring to the percentage recovery of flour from whole grain during milling. (Redrawn from Moran, R. (1959). *Nutr. Abstr. Rev.* 29:1–10.)

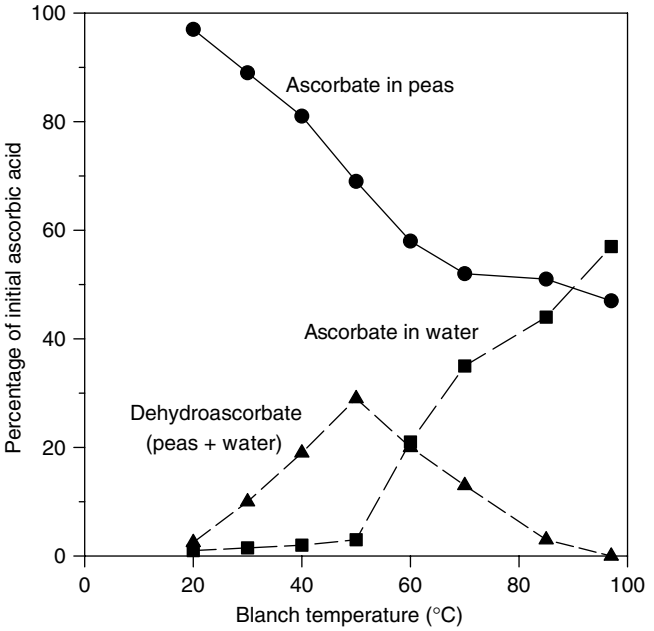


FIGURE 7.2 Retention of AA in peas during water blanching for 10 min at various temperatures. (Redrawn from Selman, J. D. (1993). *Food Chem*. 49:137–147.)

decrease interstitial gasses before further processing. Inactivation of enzymes often has a beneficial effect on the stability of many vitamins during subsequent food storage.

Blanching can be accomplished in hot water, flowing steam, hot air, or with microwaves. Blanching in hot water can cause large losses of water-soluble vitamins by leaching. An example of AA is shown in Figure 7.2. Losses of vitamins also can occur by oxidation during thermal processing following blanching. It has been well documented that high-temperature short-time (HTST) treatments improve retention of heat-labile nutrients. Specific effects of blanching have been thoroughly reviewed by Selman [128].

Changes in the vitamin content of foods during thermal processing have extensively studied and reviewed [62,63,77,123]. The elevated temperature accelerates reactions that would otherwise occur more slowly at ambient temperature. Thermally induced losses of vitamins depend on the chemical nature of the food, its chemical environment (pH, relative humidity, transition metals, other reactive compounds, concentration of dissolved oxygen, etc.), the stabilities of the individual forms of vitamins present, and the opportunity for leaching. The nutritional significance of such losses depends on the extent of loss and the importance of the food as a source of the vitamin in typical diets. Although subject to considerable variation, representative data for losses of vitamins during the canning of vegetables are shown in Table 7.6 [93].

7.6.5 Losses of Vitamins Following Processing

Compared to loss of vitamins during thermal processing, subsequent storage often has a small but significant effect on vitamin content. Several factors contribute to small postprocessing losses: (1) reaction rates are relatively slow at ambient or reduced temperature; (2) dissolved oxygen may be depleted; and (3) pH may change during processing (pH usually declines) because of thermal effects or concentrative effects (drying or freezing), and this can have a favorable effect on the stability of vitamins such as thiamin and AA. For example, Figure 7.3 illustrates vitamin C retention in canned

TABLE 7.6
Typical Losses (%) of Vitamins During Canning[a,b]

Product	Biotin	Folate	B_6	Pantothenic Acid	A	Thiamin	Riboflavin	Niacin	C
Asparagus	0	75	64	—	43	67	55	47	54
Lima beans	—	62	47	72	55	83	67	64	76
Green beans	—	57	50	60	52	62	64	40	79
Beets	—	80	9	33	50	67	60	75	70
Carrots	40	59	80	54	9	67	60	33	75
Corn	63	72	0	59	32	80	58	47	58
Mushrooms	54	84	—	54	—	80	46	52	33
Green peas	78	59	69	80	30	74	64	69	67
Spinach	67	35	75	78	32	80	50	50	72
Tomatoes	55	54	—	30	0	17	25	0	26

[a] Includes blanching.
[b] From various sources, compiled by Lund (93).

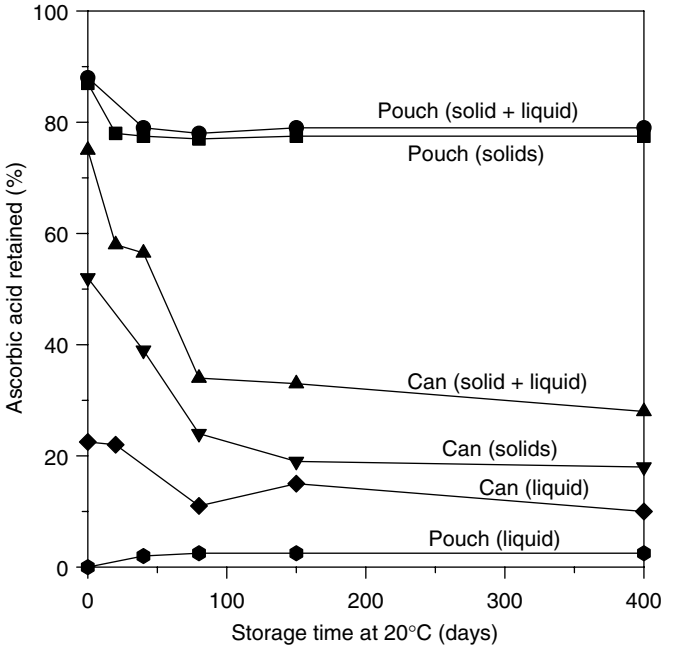


FIGURE 7.3 Retention and distribution of AA in potatoes during storage after thermal processing in cans or flexible pouches. Values show the content of AA, relative to that present prior to processing, in the potatoes and liquid of the containers. Lethality values (F_o) were not provided. (Redrawn from Ryley, J. and P. Kajda (1994). *Food Chem*. 49:119–129.)

potatoes during thermal processing. The relative importance of leaching, chemical degradation, and the type of container (cans or pouches) are apparent from these data [123].

In intermediate-moisture foods, vitamin stability is strongly influenced by water activity in addition to the other factors to be discussed. In the absence of oxidizing lipids, water-soluble vitamins generally exhibit little degradation at water activity less than or equal to monolayer hydration

(~0.2–0.3 a_w). Degradation rates increase in proportion to water activity in regions of multilayer hydration, which reflects greater solubility of the vitamin, potential reactants, and catalysts. In contrast, the influence of water activity on the stability of fat-soluble vitamins and carotenoids parallels the pattern for unsaturated fats, that is, a minimum rate at monolayer hydration and increased rates above or below this value (see Chapter 2). Substantial losses of oxidation-sensitive vitamins can occur if foods are overdried.

7.6.6 Influence of Processing Chemicals and Other Food Components

The chemical composition of food can strongly influence the stability of vitamins. Oxidizing agents directly degrade AA, folate, vitamin A, carotenoids, and vitamin E, and may indirectly affect other vitamins. The extent of their impact is dictated by concentration of the oxidant and its oxidation potential. In contrast, reducing agents such as AA and isoascorbic acid, and various thiols increase the stability of oxidizable vitamins, like tetrahydrofolates by their reducing action and as scavengers of oxygen and free radicals. The following is a brief discussion of the influence of several other processing chemicals on vitamins. See later sections for vitamin-specific details.

Chlorine is applied to foods as hypochlorous acid (HOCl), hypochlorite anion (OCl^-), molecular chlorine (Cl_2), or chlorine dioxide (ClO_2). These compounds can interact with vitamins by electrophilic substitution, by oxidation, or by chlorination of double bonds. The extent of vitamin loss caused by treatments of food with chlorinated water has not been thoroughly studied; however, one would predict relatively minor effects if the application is confined to the product surface. Chlorination of cake flour presumably has little influence on vitamins in other ingredients used in baking because residual chlorine would be negligible. Reaction products of various forms of chlorine with vitamins are, for the most part, unknown.

Sulfite and other sulfiting agents (SO_2, bisulfite, metabisulfite), as used in wines for antimicrobial effects and in dried food to inhibit enzymatic browning, have a protective effect on AA and a deleterious effect on several other vitamins. Sulfite ions directly react with thiamin causing its inactivation. Sulfite also reacts with carbonyl groups and is known to convert vitamin B_6 aldehydes (pyridoxal and pyridoxal phosphate) to their presumably inactive sulfonated derivatives. The extent to which sulfiting agents affect other vitamins has not been extensively studied.

Nitrite is used in the preservation and curing of meats and may form via microbial reduction of naturally occurring nitrate. AA or isoascorbic acid is added to nitrite-treated meats to prevent formation of *N*-nitrosamines. This is accomplished by forming NO and preventing formation of undesirable nitrous anhydride (N_2O_3, the primary nitrosating agent). The proposed reactions are shown below [84]:

$$\text{ascorbic acid} + HNO_2 \rightarrow \text{2-nitrite ester of ascorbic acid} \rightarrow \text{semidehydroascorbate radical} + NO$$

Formation of NO is desirable because it is the desired ligand for binding to myoglobin to form the cured meat color. The residual semidehydroascorbate radical retains partial vitamin C activity.

Chemical sterilants are used in highly specific applications such as treating spices with ethylene and propylene oxides for deinfestation. The biocidal function of these compounds occurs by alkylation of proteins and nucleic acids. Similar effects have been observed to occur with some vitamins, although loss of vitamin activity by this means is insignificant in the overall food supply.

Chemicals and food ingredients that influence pH will directly affect the stability of vitamins such as thiamin and AA, particularly in the neutral to mildly acidic pH range. Acidulation increases the stability of AA and thiamin. In contrast, alkalizing compounds reduce the stability of AA, thiamin, pantothenic acid, and certain folates.

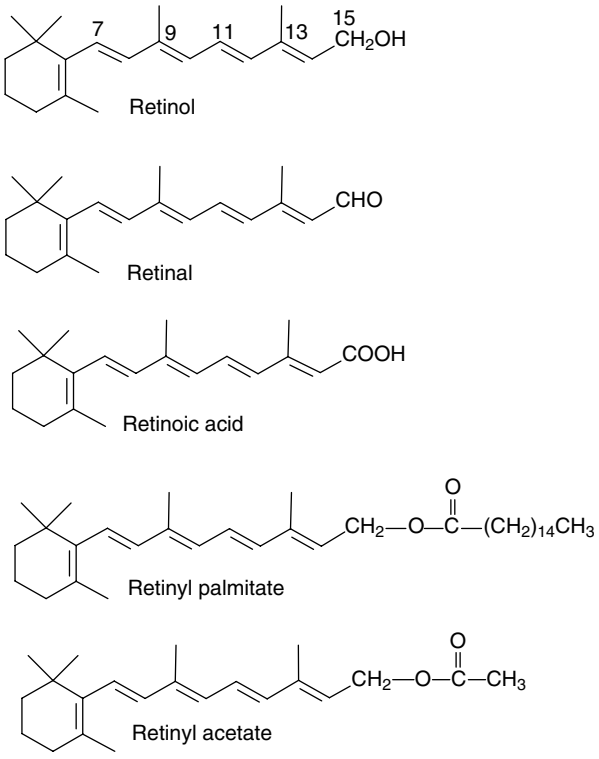


FIGURE 7.4 Structures of common retinoids.

7.7 FAT-SOLUBLE VITAMINS

7.7.1 Vitamin A

7.7.1.1 Structure and General Properties

Vitamin A refers to a group of nutritionally active unsaturated hydrocarbons, including retinol and related compounds (Figure 7.4) and certain carotenoids (Figure 7.5). Vitamin A activity in animal tissues is predominantly in the form of retinol or its esters, retinal, and to a lesser extent, retinoic acid. The concentration of vitamin A is greatest in liver, the major body pool, in which retinol and retinol esters are the primary forms present. The term retinoids refers to the class of compounds including retinol and its chemical derivatives having four isoprenoid units. Several retinoids that are analog of the nutritionally active forms of vitamin A exhibit useful pharmacological properties. In addition, synthetic retinyl acetate and retinyl palmitate are used widely in synthetic form for food fortification.

Carotenoids contribute significant vitamin A activity to foods of both plant and animal origin. Of approximately 600 known carotenoids, about 50 exhibit some provitamin A activity (i.e., are partially converted to vitamin A *in vivo*). Preformed vitamin A does not exist in plants and fungi; their vitamin A activity is associated with certain carotenoids.The structures of selected carotenoids, along with their relative vitamin A activities determined by rat bioassay and estimated Retinol Activity Equivalent values, are presented in Figure 7.5. The reader is referred to Chapter 9 for further discussion of the properties of the carotenoids in the context of their role as food pigments.

For a compound to have vitamin A or provitamin A activity, it must exhibit certain structural similarities to retinol, including: (1) at least one intact nonoxygenated β-ionone ring and (2) an isoprenoid side chain terminating in an alcohol, aldehyde, or carboxyl function (Figure 7.4).

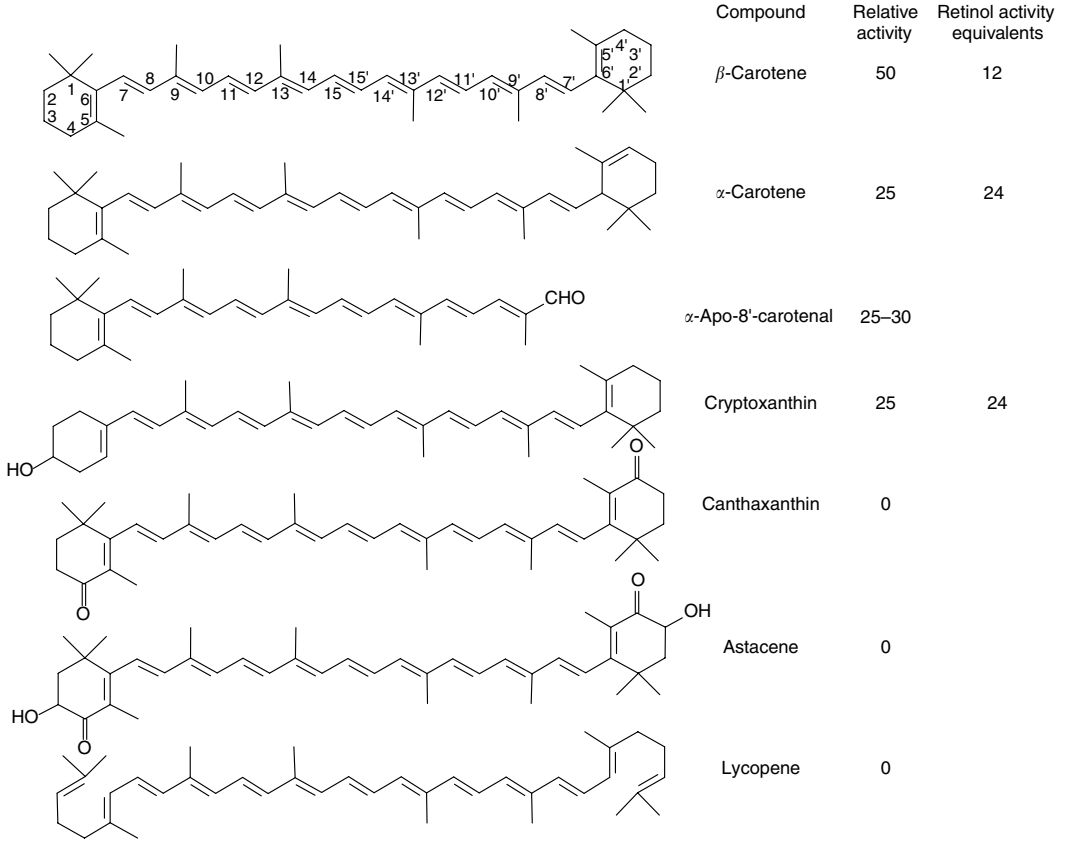


FIGURE 7.5 Structures and provitamin A activities of selected carotenoids. Relative activity values are based on an assumption of 50% for β-carotene, relative to retinol, and should be viewed as maximal estimates. Retinol Activity Equivalent values are the amount (μg) of each dietary vitamin A-active carotenoid that provides the activity of 1 μg of retinol.

The vitamin A-active carotenoids such as β-carotene (Figure 7.5) are considered to have provitamin A activity until they undergo oxidative enzymatic cleavage of the central C^{15}–$C^{15'}$ bond in the intestinal mucosa to release two molecules of active retinal. Among the carotenoids, β-carotene exhibits the greatest provitamin A activity. Carotenoids with ring hydroxylation or the presence of carbonyl groups exhibit less provitamin A activity than β-carotene if only one ring is affected, and have no activity if both rings are oxygenated. Although potentially two molecules of vitamin A are produced from each molecule of dietary β-carotene, the inefficiency of the process accounts for the fact that β-carotene exhibits only $\sim$50% of the vitamin A activity exhibited by retinol, on a mass basis. This was the basis of the initial belief that the relative vitamin A activity of retinol and β-carotene was 1:2 on a mass basis. Considerable variation exists among various animal species and humans with respect to the efficiency of utilization of carotenoids and the extent of absorption of carotenoid molecules in intact form from food sources (see discussion of bioavailability below), and some scientific disagreement exists regarding the vitamin A equivalence of β-carotene. A recent reassessment of the issues of bioavailability and bioconversion (i.e., conversion of carotenoids to vitamin A) by the U.S. Institute of Medicine has led to the recommendation that data be expressed in units of retinol activity equivalents [72]. In this system, the retinol activity equivalents for retinol, β-carotene, and other vitamin A-active carotenoids are 1:12:24 on a mass basis. For example, 12 μg β-carotene from a typical diet is now believed to yield only 1 μg retinol activity equivalent.

TABLE 7.7
Relative Vitamin A Activity of Stereoisomeric forms of Retinol Derivatives

	Relative Vitamin A Activity[a]	
Isomer	**Retinyl Acetate**	**Retinal**
All-*trans*	100	91
13-*cis*	75	93
11-*cis*	23	47
9-*cis*	24	19
9,13-di-*cis*	24	17
11,13-di-*cis*	15	31

[a] Molar vitamin A activity relative to all-*trans* retinyl acetate in rat bioassays.

Source: Ames, S. R. (1965). *Fed. Proc. Fed. Am. Soc. Exp. Biol*. 24:917–923.

In contrast to the provitamin A activity of cartenoids, the *in vivo* antioxidative function attributed to dietary carotenoids requires absorption of the intact molecule [15].

The retinoids and provitamin A carotenoids are very lipophilic compounds because of their nonpolar structures. Consequently, they associate with lipid components, specific organelles, or carrier proteins in foods and living cells. In many food systems, retinoids and carotenoids are found associated with lipid droplets or micelles dispersed in an aqueous environment. For example, both retinoids and carotenoids are present in the fat globules of milk, while in orange juice the carotenoids associate with dispersed oils. The conjugated double bond system of retinoids gives strong and characteristic ultraviolet absorption spectra, while the additional conjugated double bond system of carotenoids causes absorption in the visible spectrum and the yellow-orange color of these compounds. All-*trans* isomers exhibit the greatest vitamin A activity and are the predominant naturally occurring forms of retinoids and carotenoids in foods (Tables 7.7 and 7.8) [2,158]. Conversion to *cis* isomers, which can occur during thermal processing, causes a loss of vitamin A activity.

It is important to note that carotenoids that do not have vitamin A activity may still serve important functions in maintaining health. Analysis of tissues reveals that certain carotenoids concentrate in certain tissues, which may reflect specific antioxidative functions. Of particular interest are the roles of lycopene in the prostate and zeaxanthin and lutein in the retina. Epidemiological studies appear to support such relationships.

7.7.1.2 Stability and Modes of Degradation

The degradation of vitamin A (retinoids and vitamin A-active carotenoids) generally parallels the oxidative degradation of unsaturated lipids. Factors that promote oxidation of unsaturated lipids enhance degradation of vitamin A, either by direct oxidation or by indirect effects of free radicals. Changes in the β-carotene content of cooked dehydrated carrots illustrate typical extents of degradation during processing and typical exposure to oxygen during associated handling (Table 7.9) [27]. It should be noted, however, that extended storage of vitamin A in foods such as fortified breakfast cereal products, infant formulas, fluid milk, fortified sucrose, and condiments is usually not highly detrimental to the retention of added vitamin A.

Losses of vitamin A activity of retinoids and carotenoids in foods occur mainly through reactions involving the unsaturated isoprenoid side chain, either by autoxidation or geometric isomerization.

TABLE 7.8
Relative Vitamin A Activity of Stereoisomeric forms of Carotenes

Compound and Isomer	Relative Vitamin A Activity[a]
β-Carotene	
all-*trans*	100
9-*cis* (neo-U)	38
13-*cis* (neo-B)	53
α-Carotene	
all-*trans*	53
9-*cis* (neo-U)	13
13-*cis* (neo-B)	16

[a] Activity relative to that of all-*trans* β-carotene in rat bioassays.

Source: Zechmeister, L. (1949). *Vitam. Hormones (N. Y.)* 7:57–81.

TABLE 7.9
Concentration of β-Carotene in Cooked Dehydrated Carrots

Sample	β-Carotene Concentration (μg/g solids)
Fresh	980–1860
Explosive puff-dried	805–1060
Vacuum freeze-dried	870–1125
Conventional air-dried	636–987

Source: Dellamonica, E. S. and P. E. McDowell (1965). *Food Technol.* 19:1597–1599.

Retinoid and carotenoid molecules largely remain chemically intact during thermal processing, although they do undergo some isomerization. High-performance liquid chromatography (HPLC) analysis has revealed that many foods contain a mixture of all-*trans* and *cis* isomers of retinoids and carotenoids. As summarized in Table 7.10 [19], conventional canning of fruits and vegetables is sufficient to induce isomerization and ensuing losses of vitamin A activity. In addition to thermal isomerization, the conversion of all-*trans* forms of retinoids and carotenoids to various *cis* isomers can be induced by exposure to light, acid, chlorinated solvents (e.g., chloroform), and dilute iodine.

The occurrence of *cis* isomers of carotenoids has been known for many years (Figure 7.6). Previous nomenclature for β-carotene isomers was derived from chromatographic separations and included neo-β-carotene U (9-*cis*-β-carotene) and neo-β-carotene B (13-*cis*-β-carotene). Confusion exists in the literature because neo-β-carotene B was originally identified incorrectly as 9,13′-di-*cis*-β-carotene [145]. Analogous isomerization occurs with other carotenoids. The maximum extent of thermal isomerization generally observed in canned fruits and vegetables is about 40% 13-*cis*-β-carotene and 30% 9-*cis*-β-carotene (Table 7.10). The values observed for *cis*-isomers of β-carotene in processed foods are similar to the equilibrium values observed in the iodine-catalyzed isomerization of β-carotene, which suggests that the extent and specificity of isomerization is similar regardless of the mechanism.

Photochemical isomerization of vitamin A compounds occurs both directly and indirectly via a photosensitizer. The ratios and quantities of *cis* isomers produced differ with the means of

TABLE 7.10
Distribution of β-Carotene Isomers in Selected Fresh and Processed Fruits and Vegetables

		Percentage of Total β-Carotene		
Product	**Status**	**13-*cis***	**all-*trans***	**9-*cis***
Sweet potato	Fresh	0.0	100.0	0.0
Sweet potato	Canned	15.7	75.4	8.9
Carrot	Fresh	0.0	100.0	0.0
Carrot	Canned	19.1	72.8	8.1
Squash	Fresh	15.3	75.0	9.7
Squash	Canned	22.0	66.6	11.4
Spinach	Fresh	8.8	80.4	10.8
Spinach	Canned	15.3	58.4	26.3
Collard	Fresh	16.6	71.8	11.7
Collard	Canned	26.6	46.0	27.4
Cucumber	Fresh	10.5	74.9	14.5
Pickle	Pasteurized	7.3	72.9	19.8
Tomato	Fresh	0.0	100.0	0.0
Tomato	Canned	38.8	53.0	8.2
Peach	Fresh	9.4	83.7	6.9
Peach	Canned	6.8	79.9	13.3
Apricot	Dehydrated	9.9	75.9	14.2
Apricot	Canned	17.7	65.1	17.2
Nectarine	Fresh	13.5	76.6	10.0
Plum	Fresh	15.4	76.7	8.0

Source: Chandler, L. A. and S. J. Schwartz (1987). *J. Food Sci.* 52:669–672.

photoisomerization. Photoisomerization of all-*trans*-β-carotene involves a series of reversible reactions, and each isomerization is accompanied by photochemical degradation (Figure 7.7). Similar rates of photoisomerization and photodegradation have been observed in aqueous dispersions of β-carotene and in carrot juice. These photochemical reactions also have been observed when retinoids in foods are exposed to light (e.g., milk). The type of packaging material can have a substantial effect on net retention of vitamin A activity in food exposed to light during storage.

Oxidative degradation of vitamin A and carotenoids in foods can occur by direct peroxidation or by indirect action of free radicals produced during oxidation of fatty acids. β-Carotene, and probably other carotenoids, have the ability to act as antioxidants under conditions of reduced oxygen concentration (<0.2 atm O_2), although they may act as prooxidants at higher oxygen concentrations [15]. β-Carotene can act as an antioxidant by quenching singlet oxygen, hydroxyl and superoxide radicals, and by reacting with peroxyl radicals ($ROO^{\bullet}$). Peroxyl radicals attack β-carotene to form an adduct postulated to be ROO-β-carotene$^{\bullet}$, in which the peroxyl radical bonds to the C^7 position of β-carotene, while the unpaired electron is delocalized across the conjugated double bond system. This adduct further breaks down to form epoxide and other products. β-Carotene apparently does not act as a chain-breaking radical (donating $H^{\bullet}$) as do phenolic antioxidants. This antioxidant behavior of β-carotene, and presumably other carotenoids, causes a reduction or total loss of vitamin A activity regardless of the mechanism by which free radical initiation occurs. For retinol and retinyl esters, however, the attack of free radicals occurs mainly at the C^{14} and C^{15} positions.

Oxidation of β-carotene involves the formation of the 5,6-epoxide, which may isomerize to the 5,8-epoxide (mutachrome). Photochemically induced oxidation yields mutachrome as the primary

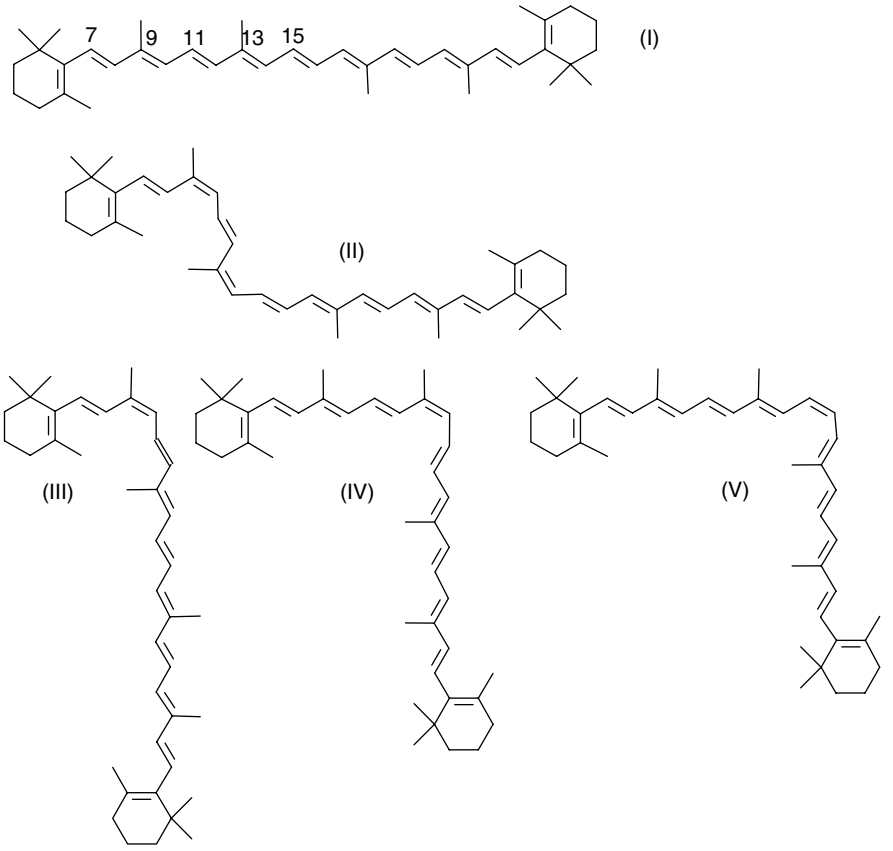


FIGURE 7.6 Structures of selected *cis* isomers of β-carotene. (I) all-*trans*; (II) 11,15-di-*cis*; (III) 9-*cis*; (IV) 13-*cis*; (V) 15-*cis*.

degradation product. Fragmentation of β-carotene to many lower molecular weight compounds can occur especially during high temperature treatments. Resulting volatiles can have a significant effect on flavor. Such fragmentation also occurs during oxidation of retinoids. An overview of these reactions and other aspects of the chemical behavior of vitamin A is shown in Figure 7.7.

7.7.1.3 Bioavailability

Retinoids are absorbed effectively except under conditions in which malabsorption of fat occurs. Retinyl acetate and palmitate are as effectively utilized as nonesterified retinol. Diets containing nonabsorbable hydrophobic materials such as certain fat substitutes may contribute to malabsorption of vitamin A. The bioavailability of vitamin A added to rice has been demonstrated in human subjects.

Aside from the inherent difference in utilization between retinol and the provitamin A carotenoids, carotenoids in many foods are poorly absorbed in the intestine. Absorption may be impaired by the specific binding of carotenoids as carotenoproteins or by entrapment in poorly digestible vegetable matrices. In studies with human subjects, β-carotene from carrots yielded only $\sim$21% of the plasma β-carotene response obtained from an equivalent dose of pure β-carotene; β-carotene in broccoli also exhibited a similar low bioavailability [12].

7.7.1.4 Analytical Methods

Early methods of vitamin A analysis centered on the reactions of retinoids with Lewis acids such as antimony trichloride and trifluoroacetic acid to yield a blue color. In addition, fluorometric methods

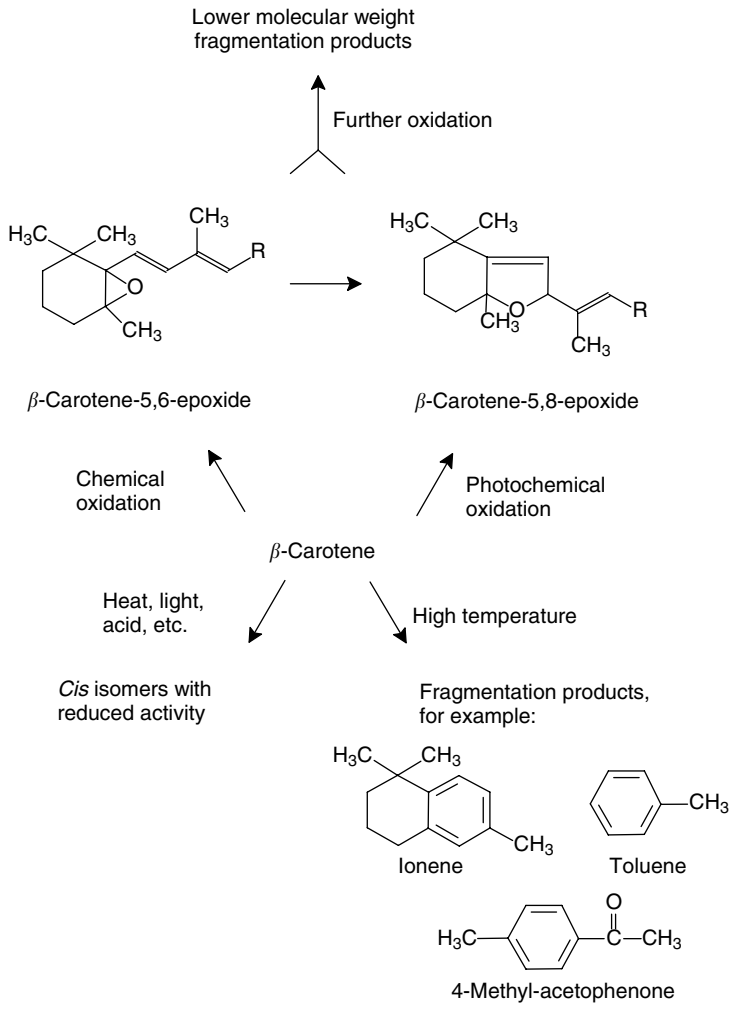


FIGURE 7.7 Overview of carotenoid degradation.

have been used to measure vitamin A [143]. Interferences often occur when these methods are applied to foods. Furthermore, these methods do not detect *trans–cis* isomerization that may occur during processing or storage of foods. Because the *cis* isomers exhibit less nutritional activity than the all-*trans* compound, it is inaccurate to equate *total* vitamin A or provitamin A activity simply as the sum of all isomeric forms. HPLC is the method of choice because it enables quantification of individual retinoids with considerable accuracy [143]. Accurate measurement of carotenoids is a very complex task in view of the many naturally occurring chemical forms present in foods [78].

7.7.2 Vitamin D

7.7.2.1 Structure and General Properties

Vitamin D activity in foods is associated with several lipid soluble sterol analogs including cholecalciferol (vitamin D_3) from animal sources and ergocalciferol (vitamin D_2) produced synthetically (Figure 7.8). Both of these compounds are used in synthetic form for food fortification. Cholecalciferol forms in human skin upon exposure to sunlight, and this is a multistep process

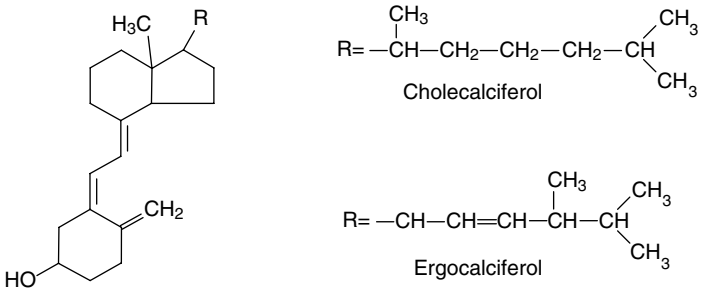


FIGURE 7.8 Structure of ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3).

involving photochemical modification of 7-dehydrocholesterol followed by nonenzymatic isomerization. Because of this *in vivo* synthesis, the requirement for dietary vitamin D will depend on the extent of exposure to sunlight. Ergocalciferol is an exclusively synthetic form of vitamin D that is formed by commercial irradiation of phytosterol (a plant sterol) with ultra violet (UV) light. Several hydroxylated metabolites of vitamin D_2 and D_3 form *in vivo*. The 1,25-dihydroxy derivative of cholecalciferol is the main physiologically active form, and it is involved in the regulation of calcium absorption and metabolism. 25-Hydroxycholecalciferol, in addition to cholecalciferol, comprises a significant amount of the naturally occurring vitamin D activity in meat and milk products.

Fortification of most fluid milk products with either ergocalciferol or cholecalciferol makes a significant contribution to dietary needs. Vitamin D is susceptible to degradation by light, and this may occur in glass-packaged milk during retail storage. For example, ~50% of cholecalciferol added to skim milk is lost during 12 days of continual exposure to fluorescent light at 4°C. It is not known whether this degradation involves direct photochemical degradation, a mechanism involving a photosensitizer yielding an active oxygen species (e.g., $^1O_2$), or as an indirect effect of light-induced lipid oxidation. Like other unsaturated fat-soluble components of foods, vitamin D compounds are susceptible to oxidative degradation. Overall, however, the stability of vitamin D in foods, especially under anaerobic conditions, is not a major concern.

7.7.2.2 Analytical Methods

Measurement of vitamin D is performed primarily by HPLC methods [147]. Alkaline conditions yield rapid degradation of vitamin D; thus, saponification as used widely in the analysis of lipid soluble materials cannot be employed. Various preparative chromatographic methods have been developed for the purification of food extracts prior to HPLC analysis.

7.7.3 Vitamin E

7.7.3.1 Structure and General Properties

Vitamin E is the generic term for tocols and tocotrienols that exhibit vitamin activity similar to that of α-tocopherol. Tocols are 2-methyl-2(4′,8′,12′-trimethyltridecyl)chroman-6-ols, while tocotrienols are identical except for the presence of double bonds at positions 3′, 7′, and 11′ of the side chain (Figure 7.9). Tocopherols, that are typically the main compounds having vitamin E activity in foods, are derivatives of the parent compound tocol, and have one or more methyl groups at positions 5′, 7′, or 8′ of the ring structure (chromanol ring) (Figure 7.9). The α, β, γ, and δ forms of tocopherol and tocotrienol differ according to the number and position of the methyl groups and thus differ significantly in vitamin E activity. The data presented in Table 7.11 represent the traditional views of the relative activities of these compounds, with α-tocopherol being the highest vitamin E activity [94]. In a new system of reporting vitamin E activity [71], α-tocopherol is viewed as the

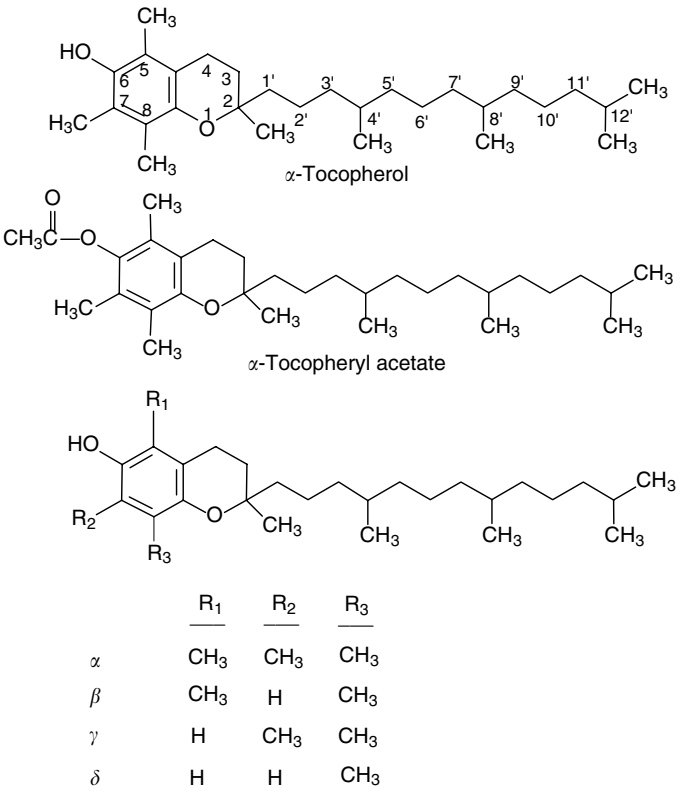


FIGURE 7.9 Structures of tocopherols. The structures of tocotrienols are identical to the corresponding tocopherols, except for the presence of double bonds at positions 3′, 7′, and 11′.

TABLE 7.11
Traditional Views of Relative Vitamin E Activity of Tocopherols and Tocotrienols

	Bioassay Method			
Compound	**Rat Fetal Resorption**	**Rat Erythrocyte Hemolysis**	**Muscular Dystrophy (Chicken)**	**Muscular Dystrophy (Rat)**
α-Tocopherol	100	100	100	100
β-Tocopherol	25–40	15–27	12	
γ-Tocopherol	1–11	3–20	5	11
δ-Tocopherol	1	0.3–2		
α-Tocotrienol	27–29	17–25		28
β-Tocotrienol	5	1–5		

Source: Machlin, L. J. (1991). Vitamin E, in *Handbook of Vitamins*, 2nd edn., *Revised and Expanded* (L. J. Machlin, ed.), Marcell Dekker, Inc., New York, pp. 99–144.

TABLE 7.12
Vitamin E Activity of Isomeric forms of α-Tocopheryl Acetate

Form of α-Tocopheryl Acetate[a]	Relative Vitamin E Activity (%)
RRR	100
All-rac	77
RRS	90
RSS	73
SSS	60
RSR	57
SRS	37
SRR	31
SSR	21

[a] R and S refer to the chiral configuration of the 2, 4′, and 8′ positions, respectively. R is the naturally occurring chiral form. All-rac signifies fully racemic.

Source: Adapted from Weiser, H. and M. Vecchi (1982). *Int. J. Vitam. Nutr. Res.* 32:351–370.

sole form exhibiting specific vitamin E activity, while α-tocopherol and all other tocopherols and tocotrienols provide a general antioxidant function. This remains an area of controversy among some researchers.

There are three asymmetric carbons (2′, 4′, and 8′) in the tocopherol molecule (Figure 7.9), and the stereochemical configuration at these positions influence the vitamin E activity of the compound. Early nomenclature for vitamin E compounds is confusing with regard to the vitamin activity of the stereoisomers. The naturally occurring configuration of α-tocopherol exhibits the greatest vitamin E activity and is now designated RRR-α-tocopherol; other terminology, such as the term D-α-tocopherol, should be discontinued.

Synthetic forms of α-tocopheryl acetate are used widely in food fortification. The presence of the acetate ester greatly improves the stability of the compound by blocking the phenolic hydroxyl group and, thus, eliminating its radical quenching activity. Synthetic forms that are racemic mixtures consisting of eight possible combinations of geometric isomers involving positions 2′, 4′, and 8′ should be designated all-rac-α-tocopheryl acetate rather than the previously used term dl-α-tocopheryl acetate. Vitamin E activity of tocopherols and tocotrienols varies according to the particular form present (α, β, γ, or δ) (Table 7.11), in addition to stereochemical nature of the tocopherol side chain (Table 7.12) [152]. The lower vitamin E activity of all-rac-α-tocopheryl acetate, relative to naturally occurring RRR-isomers of the vitamin, should be recognized and compensated for when using these compounds for food fortification. α-Tocopherol is the major form of vitamin E in most animal products, other tocopherols and tocotrienols occur in varying proportions in plant products (Table 7.13) [144,148]. A novel principle of increasing vitamin E content and vitamin activity in plants has been demonstrated which involved experimental genetic engineering of plants to increase γ-tocopherol synthesis along with increased conversion of γ-tocopherol to α-tocopherol [132].

The tocopherols and tocotrienols are very nonpolar and exist mainly in the lipid phase of foods. All tocopherols and tocotrienols, when not esterified, have the ability to act as antioxidants; they quench free radicals by donating the phenolic H^+ and an electron. Tocopherols are a natural constituents of all biological membranes and are thought to contribute to membrane stability through their antioxidant activity. Naturally occurring tocopherols and tocotrienols also contribute to the stability of highly unsaturated vegetable oils through this antioxidant action. In contrast, α-tocopheryl acetate added in

TABLE 7.13
Concentration of Tocopherols and Tocotrienols in Selected Vegetable Oils and Foods

Food	α-T	α-T3	β-T	β-T3	γ-T	γ-T3	δ-T	δ-T3
Vegetable oils (mg/100 g)								
Sunflower	56.4	0.013	2.45	0.207	0.43	0.023	0.087	
Peanut	14.1	0.007	0.396	0.394	13.1	0.03	0.922	
Soybean	17.9	0.021	2.80	0.437	60.4	0.078	37.1	
Cottonseed	40.3	0.002	0.196	0.87	38.3	0.089	0.457	
Corn	27.2	5.37	0.214	1.1	56.6	6.17	2.52	
Olive	9.0	0.008	0.16	0.417	0.471	0.026	0.043	
Palm	9.1	5.19	0.153	0.4	0.84	13.2	0.002	
Infant formula (saponified)	12.4		0.24		14.6		7.41	
Spinach	26.05	9.14						
Beef	2.24							
Wheat flour	8.2	1.7	4.0	16.4				
Barley	0.02	7.0		6.9		2.8		

Abbreviations: T, tocopherol; T3, tocotrienol.

Source: Adapted from Thompson, J. N. and G. Hatina (1979). *J. Liq. Chromatogr*. 2:327–344; Van Niekerk, P. J. and A. E. C. Burger. (1985). *J. Am. Oil Chem. Soc*. 62:531–538.

food fortification has no antioxidant activity because the acetate ester has replaced the phenolic H^+. α-Tocopheryl acetate does exhibit vitamin E activity and *in vivo* antioxidant effects as a result of enzymatic cleavage of the ester. The concentration of dietary vitamin E in animals has been shown to influence the oxidative stability of meats after slaughter. For example, the susceptibility of pork muscle products to oxidation of cholesterol and other lipids has been shown to be inversely related to α-tocopheryl acetate intake of the pigs.

7.7.3.2 Stability and Mechanism of Degradation

Vitamin E compounds exhibit reasonably good stability in the absence of oxygen and oxidizing lipids. Anaerobic treatments in food processing, such as retorting of canned foods, have little effect on vitamin E activity. In contrast, the rate of vitamin E degradation increases in the presence of molecular oxygen and can be especially rapid when free radicals are also present. Oxidative degradation of vitamin E is strongly influenced by the same factors that influence oxidation of unsaturated lipids. The a_w-dependence of α-tocopherol degradation is similar to that of unsaturated lipids, with a rate minimum occurring at the monolayer moisture value and greater rates at either higher or lower a_w (see Chapter 2). The use of intentional oxidative treatments, such as the bleaching of flour, can lead to large losses of vitamin E.

An interesting nonnutritional use of α-tocopherol in foods is in the curing of bacon to reduce formation of nitrosamines. It is thought that α-tocopherol serves as a lipid-soluble phenolic compound to quench nitrogen free radicals ($NO^{\bullet}$, $NO_2^{\bullet}$) in a radical-mediated nitrosation process.

Reactions of vitamin E compounds, especially α-tocopherol, in foods have been studied extensively. As summarized in Figure 7.10, α-tocopherol can react with a peroxyl radical (or other free radicals) to form a hydroperoxide and an α-tocopheryl radical. As with other phenolic radicals, this is relatively unreactive because the unpaired electron resonates across the phenolic ring system. Radical termination reactions can occur to form covalently linked tocopheryl dimers and trimers, while additional oxidation and rearrangement can yield tocopheroxide, tocopheryl hydroquinone,

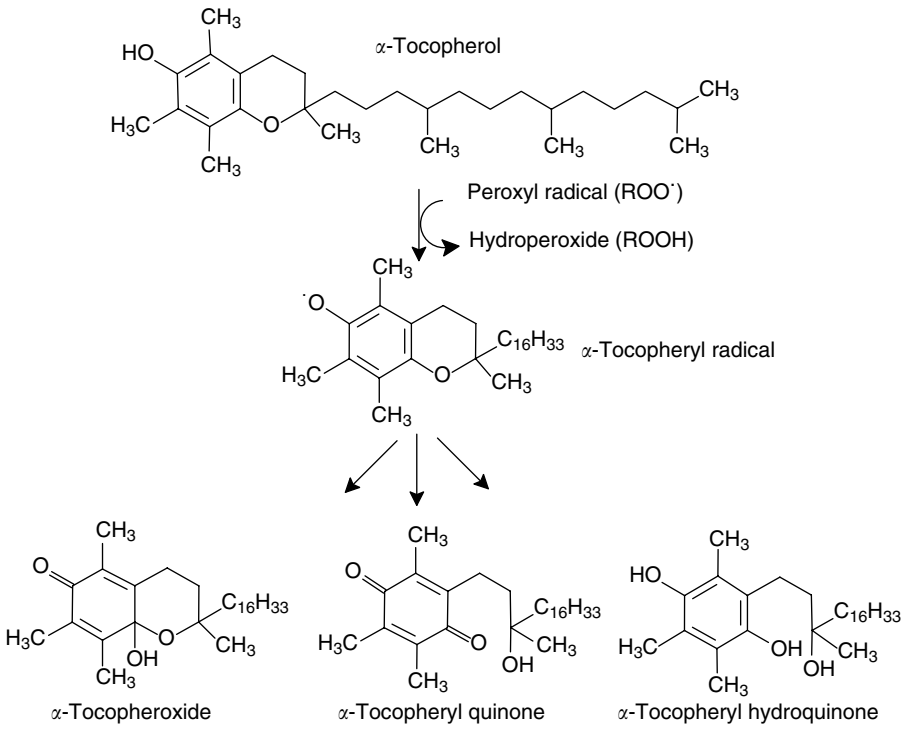


FIGURE 7.10 Overview of the oxidative degradation of vitamin E. In addition to the initial oxidation products shown, many other compounds are formed as a result of further oxidation and rearrangement.

and tocopheryl quinone (Figure 7.10). Rearrangement and further oxidation can yield many other products. Although α-tocopheryl acetate and other vitamin E esters do not participate in radical quenching, they are subject to oxidative degradation but at a lower rate than nonesterified compounds. The degradation products of vitamin E exhibit little or no vitamin activity. Through their ability to act as phenolic antioxidants, nonesterified vitamin E compounds contribute to the oxidative stability of food lipids.

Vitamin E compounds also can contribute indirectly to oxidative stability of other compounds by scavenging singlet oxygen while being concurrently degraded. As shown in Figure 7.11, singlet oxygen directly attacks the tocopherol molecule ring system to form a transient hydroperoxydieneone derivative. This can rearrange to form both the tocopheryl quinone and the tocopheryl quinone 2,3-oxide which have little vitamin E activity. The order of reactivity toward singlet oxygen is $\alpha > \beta > \gamma > \delta$, and antioxidative potency is in the reverse order. Tocopherols also can physically quench singlet oxygen, which involves deactivation of the singlet state oxygen without oxidation of the tocopherol. These attributes of tocopherols are consistent with the fact that tocopherols are potent inhibitors of photosensitized, singlet-oxygen-mediated oxidation of soybean oil.

7.7.3.3 Bioavailability

The bioavailability of vitamin E compounds is usually quite high in individuals who digest and absorb fat normally. On a molar basis, bioavailability of α-tocopheryl acetate is nearly equivalent to that of α-tocopherol [16] except at high doses where the enzymatic deesterification of α-tocopheryl acetate can be limiting. Previous studies which indicated that α-tocopheryl acetate was more potent than α-tocopherol on a molar basis may have been biased by the susceptibility of α-tocopherol to undergo oxidation prior to testing.

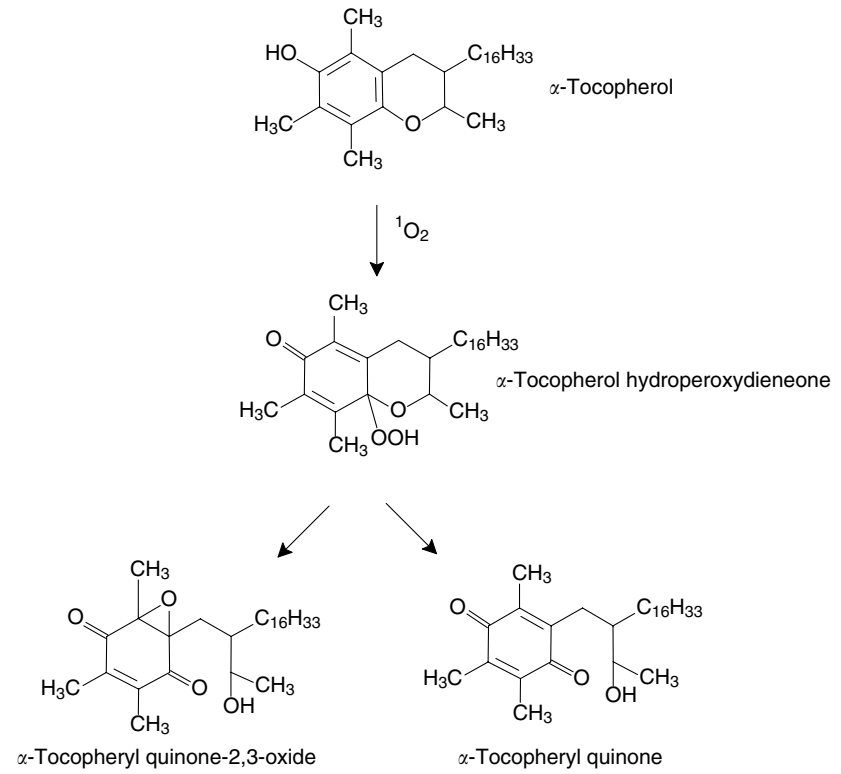


FIGURE 7.11 Reaction of singlet oxygen and α-tocopherol.

7.7.3.4 Analytical Methods

HPLC methods for determination of vitamin E have largely superseded previous spectrophotometric and direct fluorometric procedures. The use of HPLC permits the measurement of specific forms of vitamin E (e.g., α-, β-, γ-, and δ-tocopherols and tocotrienols) and, thus, estimation of total vitamin E activity in a product based on relative potencies of the specific compounds [22]. Detection can be accomplished using either UV absorbance or fluorescence. When saponification is used to aid in separation of lipids from vitamin E, any vitamin E ester will be hydrolyzed to free α-tocopherol. Care must be taken to prevent oxidation during extraction, saponification, and other preliminary treatments.

7.7.4 Vitamin K

7.7.4.1 Structure and General Properties

Vitamin K consists of a group of naphthoquinones that exist with or without a terpenoid side chain in the 3-position (Figure 7.12). The unsubstituted form of vitamin K is menadione, and it is of primary significance as a synthetic form of the vitamin that is used in vitamin supplements and food fortification. Phylloquinone (vitamin K_1) is a product of plant origin, while menaquinones (vitamin K_2) of varying chain length are products of bacterial synthesis, mainly by intestinal microflora. Phylloquinones occur in relatively large quantities in leafy vegetables including spinach, kale, cauliflower, and cabbage, and they are present, but less abundant, in tomatoes and certain vegetable oils. Vitamin K deficiency is rare in healthy individuals because of the widespread presence of phylloquinones in the diet and because microbial menoquinones are absorbed from the lower

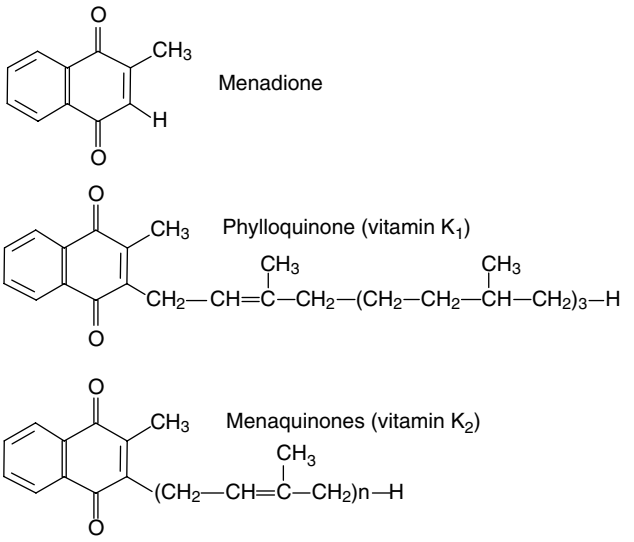


FIGURE 7.12 Structure of various forms of vitamin K.

intestine. Vitamin K deficiency is ordinarily associated with malabsorption syndromes or the use of pharmacological anticoagulants. Although the use of certain fat substitutes has been reported to impair vitamin K absorption, moderate intakes of these substitutes have no significant effect on vitamin K utilization.

The quinone structure of vitamin K compounds can be reduced to the hydroquinone form by certain reducing agents, but vitamin K activity is retained. Photochemical degradation can occur, but the vitamin is quite stable to heat.

7.7.4.2 Analytical Methods

Spectrophotometric and chemical assays based on measurement of oxidation–reduction properties of vitamin K lack the specificity required for food analysis. Various HPLC methods exist that provide satisfactory specificity and permit individual forms of vitamin K to be measured [147].

7.8 WATER-SOLUBLE VITAMINS

7.8.1 Ascorbic Acid

7.8.1.1 Structure and General Properties

L-Ascorbic acid (L-AA) (Figure 7.13) is a carbohydrate-like compound whose acidic and reducing properties are contributed by the 2,3-enediol moiety. This compound is highly polar; thus, it is readily soluble in aqueous solution and insoluble in less nonpolar solvents. AA is acidic in character as a result of ionization of the C-3 hydroxyl group ($pK_{a1} = 4.04$ at 25°C). A second ionization, dissociation of the C-2 hydroxyl, is much less favorable ($pK_{a2} = 11.4$).

AA contains two optically active centers at the C4 and C5 positions. L-Isoascorbic acid, the C-5 optical isomer, and D-AA, the C-4 optical isomer (Figure 7.13), behave in a chemically similar manner to AA, but these compounds have essentially no vitamin C activity. L-Isoascorbic acid (also known as erythorbic acid) and AA are widely used as food ingredients for their reducing and antioxidative activity (e.g., in the curing of meats and for inhibiting enzymatic browning in fruits and vegetables), but isoascorbic acid (and D-AA) has no nutritional value.

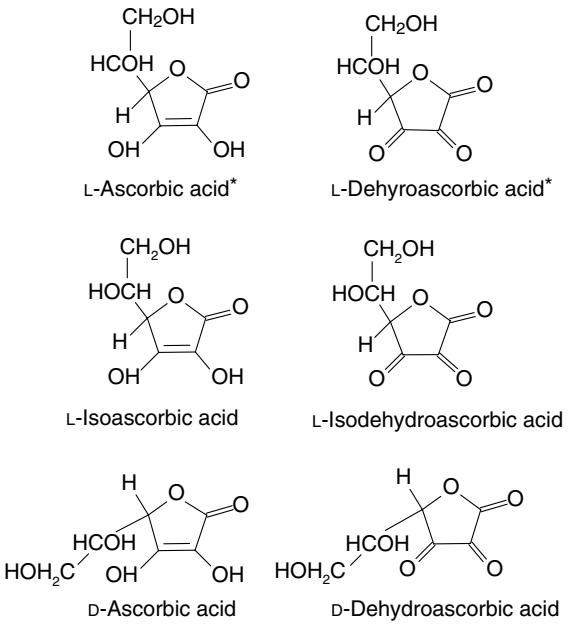


FIGURE 7.13 Structures of L-AA and L-DHAA and their isomeric forms. (* indicates vitamin C activity).

AA occurs naturally in fruits and vegetables and, to a lesser extent, in animal tissues and animal-derived products. It occurs naturally almost exclusively in the reduced L-AA (i.e., AA) form. Two-electron oxidation and hydrogen dissociation convert L-AA to L-dehydroascorbic acid (DHAA). DHAA exhibits approximately the same vitamin activity as AA because it is almost completely reduced to AA in the body. The concentration of DHAA found in foods is almost always substantially lower than AA and is a function of the rates of ascorbate oxidation and DHAA hydrolysis to 2,3-diketogulonic acid. Dehydroascorbate reductase and ascorbate free radical reductase activity exist in certain animal tissues. These enzymes are believed to conserve the vitamin through recycling and contribute to low DHAA concentrations. A significant but currently unknown fraction of the DHAA in foods and biological materials appears to be an analytical artifact that arises from oxidation of AA to DHAA during sample preparation and analysis. The instability of DHAA further complicates this analysis.

AA may be added to foods as the undissociated acid or as the neutralized sodium salt (sodium ascorbate). Conjugation of AA with hydrophobic compounds confers lipid solubility to the AA moiety. Fatty acid esters such as ascorbyl palmitate and AA acetals (Figure 7.14) are lipid soluble and can provide a direct antioxidative effect in lipid environments.

Oxidation of AA takes place as either a two one-electron transfer process or as a single two-electron reaction without detection of the semidehydroascorbate intermediate (Figure 7.15). In one-electron oxidations, the first step involves transfer of an electron to form the free radical semi DHAA. Loss of an additional electron yields DHAA, which is highly unstable because of the susceptibility to hydrolysis of the lactone bridge. Such hydrolysis, which irreversibly forms 2,3-diketogulonic acid (Figure 7.16), is responsible for loss of vitamin C activity.

AA is highly susceptible to oxidation, especially when catalyzed by transition metal ions such as Cu^{2+} and Fe^{3+}. Heat and light also accelerate the process, while factors such as pH, oxygen concentration, and water activity strongly influence the rate of reaction. Since hydrolysis of DHAA occurs very readily, oxidation to DHAA represents an essential and frequently rate-limiting step of the oxidative degradation of vitamin C.

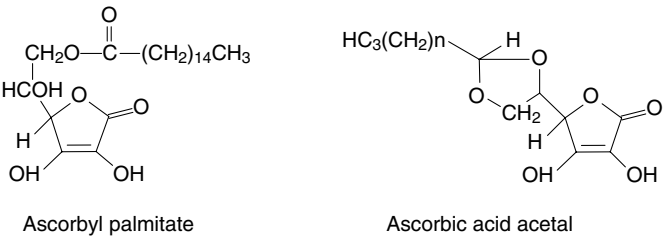


FIGURE 7.14 Structures of ascorbyl palmitate and acetals.

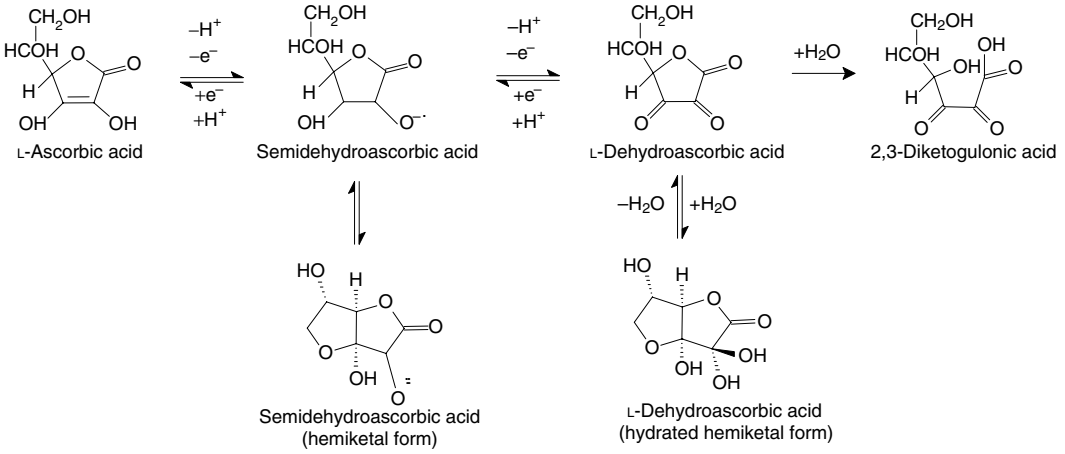


FIGURE 7.15 Sequential one-electron oxidations of L-AA. All have vitamin C activity except 2,3-diketogulonic acid.

A frequently overlooked property of AA is its ability, at low concentrations, to act as a prooxidant with high oxygen tension. Presumably this occurs by ascorbate-mediated generation of hydroxyl radicals ($OH^{\bullet}$) or other reactive species. This appears to be of minor importance in most aspects of food chemistry.

7.8.1.2 Stability and Modes of Degradation

7.8.1.2.1 Overview

Because of the high solubility of AA aqueous solutions, the potential exists for significant losses by leaching from freshly cut or bruised surfaces of fruits and vegetables. Chemical degradation primarily involves oxidations to DHAA, followed by hydrolysis to 2,3-diketogulonic acid and further oxidation, dehydration, and polymerization to form a wide array of other nutritionally inactive products. The oxidation and dehydration processes closely parallel dehydration reactions of sugars that lead to many unsaturated products and polymers (Figure 7.16).

Foods can undergo large losses of AA during routine storage and handling, including frozen storage. For example, loss of AA in commercially packed frozen green peas, spinach, green beans and okra follows first-order kinetics in the temperature range -5 to $-20°C$, with temperature dependence according to the Arrhenius equation [46]. However, the stability of AA was least in spinach ($t^{1/2}$ = 8–155 d for -5 to $-20°C$) and greatest in okra ($t^{1/2}$ = 40–660 d at -5 to $-20°C$). These findings illustrate that AA stability is dependent on food composition in addition to storage conditions. Thus, the rate of AA degradation determined for one type of food cannot necessarily be used

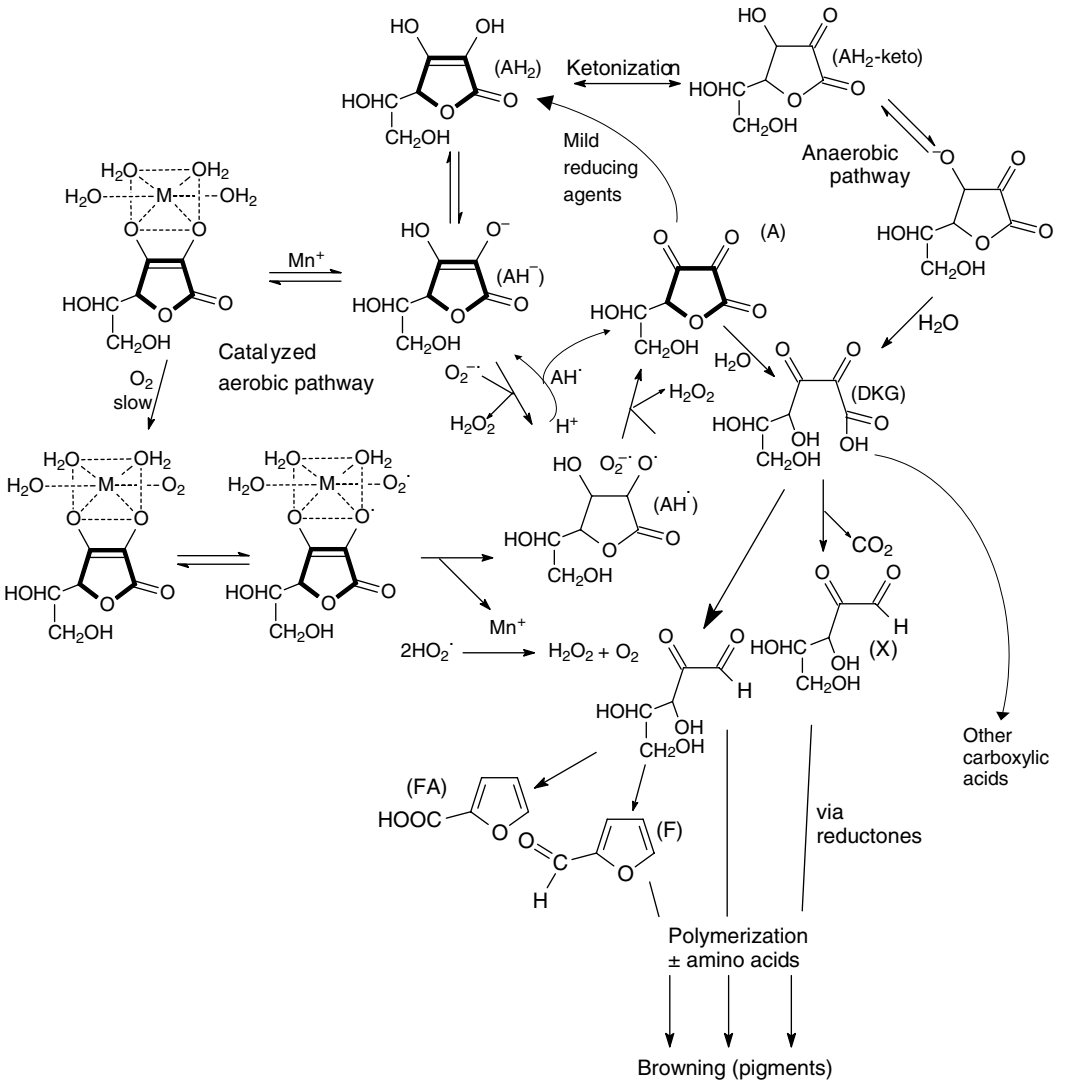


FIGURE 7.16 Overview of mechanisms for the oxidative and anaerobic degradation of AA. Structures with bold lines are primary sources of vitamin C activity. Abbreviations: AH_2, fully protonated ascorbic acid; AH^-, ascorbate monoanion; $AH^{\bullet}$, semidehydroascorbate radical; A, dehydroascorbic acid; FA, 2-furoic acid; F, 2-furaldehyde; DKG, diketogulonic acid; DP, 3-deoxypentosone; X, xylosone; Mn^+, metal catalyst; $HO_2^{\bullet}$, perhydroxyl radical. (Based on Buettner, G. R. (1988). *J. Biochem. Biophys. Methods* 16:27–40; Buettner, G. R. (1993). *Arch. Biochem. Biophys.* 300:535–543; Khan, M. M. T. and A. E. Martell (1967). *J. Am. Chem. Soc.* 89:4176–4185; Khan, M. M. T. and A. E. Martell. (1967). *J. Am. Chem. Soc*. 89:7104–7111; Liao, M.-L. and P. A. Seib (1987). *Food Technol*. 41:104–107, 111; and Tannenbaum, S. R., et al. (1985). Vitamins and minerals, in *Food Chemistry, 2nd Edition, Revised and Expanded* (O. R. Fennema, ed.), Marcell Dekker, New York, pp. 477–544.)

to predict the kinetics of AA degradation in another food system even if only subtle differences in composition exist.

The rate of oxidative degradation of the vitamin is a nonlinear function of pH because the various ionic forms of the AA differ in their susceptibility to oxidation: fully protonated (AH_2) < ascorbate monoanion (AH^-) < ascorbate dianion (A^{2-}) [16]. Under conditions relevant to most foods, pH

dependence of oxidation is governed mainly by the relative concentration of AH_2 and AH^- species, and this, in turn, is governed by pH (pK_{a1} 4.04). The presence of significant concentrations of the A^{2-} form, as controlled by pK_{a2} of 11.4, yields an increase in rate at pH $\geq$8.

7.8.1.2.2 Catalytic Effects of Metal Ions

The overall scheme of AA degradation depicted in Figure 7.5 is an integrated view of the effects of metal ions and the presence or absence of oxygen on the mechanism of AA degradation. The rate of oxidative degradation of AA is generally observed to be first order with respect to the concentration of the AH^-, molecular oxygen, and the metal ion. It was once believed that oxidative degradation of AA at neutral pH and in the absence of metal ions (i.e., the "uncatalyzed" reaction) occurred at a rate that was slow but significant. For example, a first-order rate constant of 5.87×10^{-4}/sec has been reported for the assumed spontaneous uncatalyzed oxidation of ascorbate at neutral pH. However, later evidence indicates a much smaller rate constant of 6×10^{-7}/sec for AA oxidation in an air-saturated solution at pH 7.0 [13]. This difference suggests that uncatalyzed oxidation is essentially negligible and that trace metals in foods or experimental solutions are responsible for much of the oxidative degradation. Rate constants obtained in the presence of metal ions at concentrations of several ppm are several orders of magnitude greater than those obtained in solutions nearly devoid of metal ions.

The rate of metal-catalyzed oxidation of AA is proportional to the partial pressure of dissolved oxygen over the range of 1.0–0.4 atm, and is independent of oxygen concentration at partial pressures <0.20 atm [79]. In contrast, the oxidation of AA catalyzed by metal chelates is independent of oxygen concentration [80].

The potency of metal ions in catalyzing ascorbate degradation depends on the metal involved, its oxidation state, and the presence of chelators. Catalytic potency is as follows: Cu(II) is about 80 times more potent than Fe(III), and the chelate of Fe(III) and ethylenediaminetetraacetic acid (EDTA) is ~4 times more catalytic than free Fe(III) [13]. When the rate expression of total ascorbate oxidation (TA) is presented as:

$$-\mathrm{d}\frac{[\mathrm{TA}]}{\mathrm{d}t} = k_{\mathrm{cat}} * [\mathrm{AH}^-] * [\mathrm{Cu(II)\ or\ Fe(III)}],$$

the metal ion concentration and the k_{cat} for metal ions can be used to estimate the rate AA degradation (where [TA] = concentration of total AA = [AA + DHAA] and [AH^-] is the concentration of the AH^-). In pH 7.0 phosphate buffers (20°C), k_{cat} values for Cu(II) and Fe(III) are 880 and 42 (M^{-1}/sec), respectively. It should be noted that the relative and absolute values of these catalytic rate constants in simple solutions may differ from those of actual food systems. This is likely because trace metals may associate with other constituents (e.g., amino acids) or may participate in other reactions, some of which may generate reactive free radicals or active oxygen species that may hasten oxidation of AA.

In contrast to the enhanced catalytic potency of Fe(III) when chelated by EDTA, Cu(II)-catalyzed oxidation of ascorbate is largely inhibited in the presence of EDTA [13]. Thus, the influence of EDTA or other chelators (e.g., citrate and polyphosphates) on the oxidation of AA in foods is not fully predictable.

7.8.1.2.3 Mechanisms of AA Degradation

The mechanism of AA degradation may differ depending on the nature of the food system or reaction medium. Metal-catalyzed degradation of AA has been proposed to occur through formation of a ternary complex of ascorbate monoanion, O_2, and a metal ion (Figure 7.16). The ternary complex of ascorbate, oxygen, and metal catalyst appears to yield directly DHAA as the product, without detectable formation of the product of one-electron oxidation, semidehydroascorbate radical. Alternatively, AA degradation may be initiated by a variety of one-electron oxidations. As reviewed by

TABLE 7.14
Reduction Potential of Selected Free Radicals and Antioxidants Arranged from the Most Highly Oxidizing (Top) to the Most Highly Reducing. Each Oxidized Species in an Oxidation–Reduction Couple is Capable of Abstracting an Electron or H Atom from Any Reduced Species Below It

Couple[a]		
Oxidized	**Reduced**	**$\Delta E^{o'}$ (mV)**
$HO^{\bullet}$, H^+	H_2O	2310
$RO^{\bullet}$, H^+	ROH	1600
$HO_2^{\bullet}$, H^+	H_2O_2	1060
$O_2^{-\bullet}$, $2H^+$	H_2O_2	940
RS	RS^-	920
O_2 ($^1\Delta_g$)	$O_2^{-\bullet}$	650
$PUFA^{\bullet}$, H^+	PUFA-H	600
α-Tocopheroxyl$^{\bullet}$, H^+	α-Tocopherol	500
H_2O_2, H^+	H_2O, $OH^{\bullet}$	320
Ascorbate$^{-\bullet}$, H^+	Ascorbate monoanion	282
Fe(III)EDTA	Fe(II)EDTA	120
Fe(III)aq	Fe(II)aq	110
Fe(III)citrate	Fe(II)citrate	~100
Dehydroascorbate	Ascorbate$^{-\bullet}$	~100
Riboflavin	Riboflavin$^{-\bullet}$	−317
O_2	$O_2^{-\bullet}$	−330
O_2, H^+	$HO_2^{\bullet}$	−460

[a] Nomenclature: ascorbate$^{-\bullet}$, semidehydroascorbate radical; PUFA, polyunsaturated fatty acid radical; PUFA-H, polyunsaturated fatty acid, *bis*-allylic H; $RO^{\bullet}$, aliphatic alkoxy radical. $\Delta E^{o'}$ is the standard one-electron reduction potential (mV).

Source: Adapted from Buettner, G. R. (1993). *Arch. Biochem. Biophys.* 300:535–543.

Buettner [14], there are many ways in which the one-electron oxidation of AH^- to $A^{-\bullet}$ and $A^{-\bullet}$ to form DHAA can occur. A ranking of the reduction potential, that is, reactivity, of relevant oxidants is summarized in Table 7.14. This illustrates the interrelationships in antioxidative function of several vitamins including AA, α-tocopherol, and riboflavin.

The loss of vitamin C activity during oxidative degradation of AA occurs with the hydrolysis of the DHAA lactone to yield 2,3-diketogulonic acid. This hydrolysis is favored by alkaline conditions; DHAA is most stable at pH 2.5–5.5, and its stability decreases as pH increases. For example, half-time values for DHAA hydrolysis at 23°C are 100 and 230 min at pH 7.2 and 6.6, respectively [10]. The rate of DHAA hydrolysis markedly increases with increasing temperature but is unaffected by the presence or absence of oxygen. In view of the labile nature of DHAA at neutral pH, analytical data showing significant quantities of DHAA in foods should be viewed with caution because elevated DHAA concentrations may also reflect uncontrolled oxidation during the analysis.

Although the ternary complex, as proposed by Khan and Martell [79], is apparently an accurate model of AA oxidation, later findings have expanded our knowledge of the mechanism. Scarpa et al. [127] observed that metal-catalyzed oxidation of the ascorbate monoanion (AH^-) forms superoxide ($O_2^{-\bullet}$) in the rate-determining step:

$$AH^- + O_2 \xrightarrow{\text{catalyst}} AH^{\bullet} + O_2^{-\bullet}$$

Subsequent steps of the reaction involve superoxide as a rate enhancer, effectively doubling the overall rate of ascorbate oxidation to form dehydroascorbate (A) through:

$$AH^- + O_2^{-\bullet} \xrightarrow{2H^+} AH^\bullet + H_2O_2$$

$$AH^\bullet + O_2^{-\bullet} \xrightarrow{H^+} A + H_2O_2$$

As shown in Figure 7.16, a termination-like reaction also can occur which involves two ascorbate radicals as:

$$2AH^\bullet \xrightarrow{-H^+} A + AH^-$$

Anaerobic degradation of AA (Figure 7.16) is relatively insignificant as a means of loss of the vitamin in most foods. The anaerobic pathway becomes most significant in canned products, for example, vegetables, tomatoes, and fruit juices after depletion of residual oxygen, but even in these products loss of AA through anaerobic means typically occurs very slowly. Surprisingly, the pathway not requiring oxygen (i.e., anaerobic pathway) has been identified as the predominant mechanism for loss of AA during storage of dehydrated tomato juice in the presence or absence of oxygen. Trace-metal catalysis of anaerobic degradation has been demonstrated, with the rate increasing in proportion to copper concentration.

The mechanism of anaerobic degradation of AA has not been fully established. Direct cleavage of the 1,4-lactone bridge *without* prior oxidation to DHAA appears to be involved, perhaps following an enol–keto tautomerization as shown in Figure 7.16. Unlike degradation of AA under oxidative conditions, anaerobic degradation exhibits a maximum rate at pH ~3–4. This maximum rate in the mildly acidic range may reflect the effects of pH on the opening of the lactone ring and on the concentration of the monoanionic ascorbate species.

The complexity of the anaerobic degradation mechanism, and the influence of food composition, is suggested by the significant change in activation energy at 28°C for the loss of total vitamin C in single strength orange juice during storage. In contrast, the Arrhenius plot for the degradation of total vitamin C during the storage of canned grapefruit juice is linear over the same range (4–50°C), which suggests that a single mechanism predominates [105]. The reason for this kinetic or mechanistic difference in such similar products is not known.

In view of the existence of residual oxygen present in many food packages, degradation of AA in sealed containers, especially cans and bottles, would typically occur by both oxidative and anaerobic pathways. In most cases, rate constants for anaerobic degradation of AA will be 2–3 orders of magnitude less than those for the oxidative reaction.

7.8.1.2.4 Products of AA Degradation

Regardless of the mechanism of degradation, opening of the lactone ring irreversibly destroys vitamin C activity. Although lacking in nutritional relevance, the many reactions involved in the terminal phases of ascorbate degradation are important because of their involvement in producing flavor compounds or precursors and through their participation in nonenzymatic browning.

Over 50 low molecular weight products of AA degradation have been identified. The kinds and concentrations of such compounds, and the mechanisms involved are strongly influenced by factors such as the temperature, pH, water activity, concentrations of oxygen and metal catalysts, and presence of active oxygen species. Three general types of decomposition products have been identified: (1) polymerized intermediates; (2) unsaturated carboxylic acids of 5- and 6-carbon chain length; and (3) fragmentation products having five or fewer carbons. Generation of formaldehyde during thermal degradation of ascorbate at neutral pH also has been reported. Some of these compounds are likely contributors to the changes in flavor and odor that occur in citrus juices during storage or excessive processing.

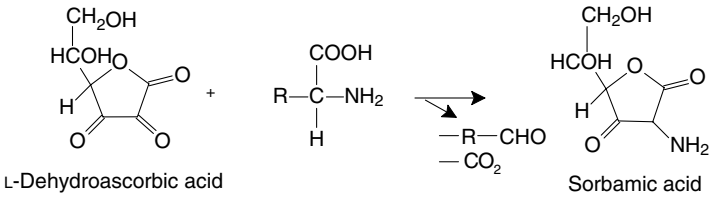


FIGURE 7.17 Participation of dehydroascorbic acid in the Strecker degradation reaction.

The degradation of sugars and AA is strikingly similar, and in some cases mechanistically identical. Qualitative differences between aerobic and anaerobic conditions occur in the pattern of AA degradation, and pH exerts an influence in all circumstances. The major AA breakdown products in neutral and acidic solution include: L-xylosone; oxalic acid; L-threonic acid; tartaric acid; 2-furaldehyde (furfural); and furoic acid, as well as a wide variety of carbonyls and other unsaturated compounds. As with sugar degradation, the extent of fragmentation increases under alkaline conditions.

AA degradation is associated with discoloration reactions in both the presence and absence of amines. DHAA, as well as the dicarbonyls formed during its degradation, can participate in Strecker degradation with amino acids. Following Strecker degradation of DHAA with an amino acid, the sorbamic acid product (Figure 7.17) can form dimers, trimers, and tetramers, several of which are reddish or yellowish in color [13,14,79,80,90,140]. In addition, 3,4-dihydroxy-5-methyl-2(5H)-furanone, an intermediate product of dehydration following decarboxylation during anaerobic degradation of AA, has a brownish color. Further polymerization of these or other unsaturated products forms either melanoidins (nitrogenous polymers) or nonnitrogenous caramel-like pigments. Although the nonenzymatic browning of citrus juices and related beverages is a complex process, the contribution of AA to browning has been clearly demonstrated [74].

7.8.1.2.5 Other Environmental Variables

Aside from the factors affecting ascorbate stability as discussed previously, many other variables influence retention of this vitamin in foods. As with many other water soluble compounds, the rate of oxidation of AA in low-moisture food systems simulating breakfast cereals has been found to increase progressively over the range of ~0.10–0.65 water activity [29,75,86] (Figure 7.18). This apparently is associated with increased availability of water to act as a solvent for reactants and catalysts. The presence of certain sugars (ketoses) can increase the rate of anaerobic degradation. Sucrose has a similar effect at low pH, consistent with its pH-dependent generation of fructose. In contrast, some sugars and sugar alcohols exert a protective effect against the oxidative degradation of AA, possibly by binding metal ions and reducing their catalytic potency. Adverse effects of photosensitizing agents on AA retention occur through the generation of singlet oxygen. The significance of these observations to actual foods remains to be determined.

7.8.1.3 Functions of AA in Foods

In addition to its function as an essential nutrient, AA is widely used as a food ingredient/additive because of its reducing and antioxidative properties. As discussed elsewhere in this book, AA effectively inhibits enzymatic browning primarily by reducing orthoquinone products. Other functions include: (1) reductive action in dough conditioners; (2) protection of certain oxidizable compounds (e.g., folates) by reductive effects, free radical scavenging, and oxygen scavenging; (3) inhibition of nitrosamine formation in cured meats; and (4) reduction of metal ions.

The antioxidative role of AA is multifunctional, with ascorbate inhibiting lipid autoxidation by several mechanisms [14,90,133]. These include: (1) scavenging singlet oxygen; (2) reduction

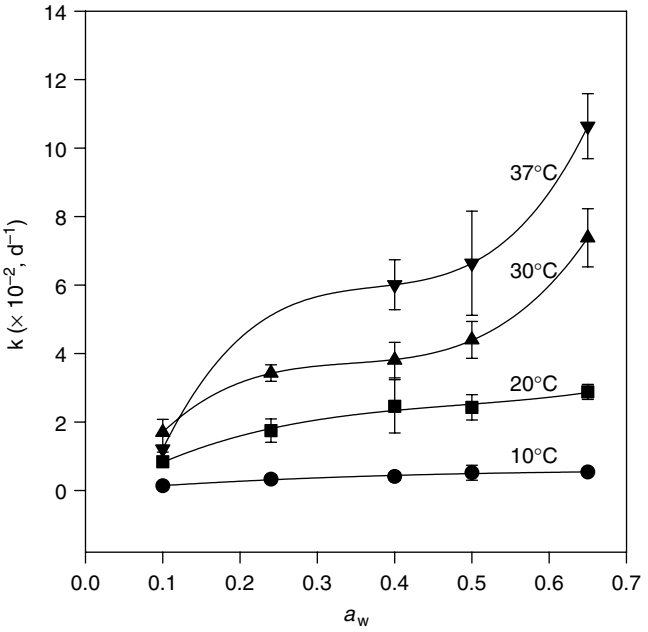


FIGURE 7.18 Degradation of ascorbic acid as a function of storage temperature and water activity in dehydrated model food systems simulating breakfast cereal products. Data (means ± SD) are expressed as apparent first-order rate constants for the loss of total ascorbic acid (AA + DHAA). (Kirk, J., et al. (1977). *J. Food Sci.* 42:1274–1279.)

of oxygen- and carbon-centered radicals, with formation of a less reactive semidehydroascorbate radical or DHAA; (3) preferential oxidation of ascorbate, with concurrent depletion of oxygen; and (4) regeneration of other antioxidants, for example, through reduction of the tocopherol radical.

AA is a very polar compound and is essentially insoluble in oils. However, AA is surprisingly effective as an antioxidant when dispersed in oils as well as in emulsions [43]. Combinations of AA and α-tocopherol are especially effective in bulk oil-based systems, while the combination of α-tocopherol and the lipophilic ascorbyl palmitate is more effective in oil-in-water emulsions. Similarly, ascorbyl palmitate has been shown to act synergistically with α-tocopherol and other phenolic antioxidants.

7.8.1.4 Bioavailability of AA in Foods

The principal dietary sources of AA are fruits, vegetables, juices, and fortified foods (e.g., breakfast cereals). The bioavailability of AA in cooked broccoli, orange sections, and orange juice has been shown to be equivalent to that of vitamin-mineral tablets for human subjects [96]. Bioavailability of AA in raw broccoli is 20% lower than that in cooked broccoli. This may be caused by incomplete disruption of cells during chewing and digestion. The relatively small difference in AA bioavailability in raw broccoli and potentially other raw vegetables, relative to their cooked forms, may have little nutritional significance. Overall, it is clear that AA in most fruits and vegetables is highly available to humans [49,71].

7.8.1.5 Analytical Methods

Many procedures exist for the measurement of AA in foods, and selection of a suitable analytical method is essential to obtain accurate results [108]. AA absorbs UV light strongly ($\lambda_{max} \sim 245$ nm),

although direct spectrophotometric analysis is precluded by the many other chromophores found in most foods. DHAA absorbs only weakly at its $\lambda_{max} \sim 300$ nm. Traditional analytical procedures involve redox titration of the sample with a dye such as 2,6-dichlorophenolindophenol, during which oxidation of AA accompanies reduction of the dye to its colorless form. A limitation of this approach is the interference by other reducing agents and the lack of a response to DHAA. Sequential analysis of the sample before and after saturation with H_2S gas or treatment with a thiol reagent to reduce DHAA to L-AA permits the measurement of total AA. Measurement of DHAA by difference lacks the precision of direct analysis, however.

An alternative approach involves condensation of DHAA (formed by controlled oxidation of L-AA in the sample) with various carbonyl reagents. Direct treatment with phenylhydrazine to form the spectrophotometrically detectable ascorbyl-*bis*-phenylhydrazone derivative permits the simple measurement of L-AA in pure solution. Many carbonyl compounds in foods will interfere with this procedure. A similar method involves the reaction of DHAA with *o*-phenylenediamine, which forms a tricyclic highly fluorescent condensation product. Although more specific and sensitive than the phenylhydrazine method, the *o*-phenylenediamine procedure is also subject to interference by certain dicarbonyls in foods. Foods containing isoascorbic acid cannot be analyzed for vitamin C by redox titration or condensation with carbonyl reagents because these methods respond to this nutritionally inactive compound.

Many HPLC methods permit accurate and sensitive measurement of total AA (before and after treatment with a reducing agent), and certain methods permit direct measurement of L-AA and DHAA. The coupling of chromatographic separation with spectrophotometric, fluorometric, or electrochemical detection makes HPLC analysis far more specific than traditional redox methods. HPLC methods have been reported that permit the simultaneous determination of ascorbic and isoascorbic acids as well as their dehydro forms [146]. A method based on gas chromatography–mass spectrometry has been reported, but extensive sample preparation is a disadvantage of the procedure [31].

7.8.2 Thiamin

7.8.2.1 Structure and General Properties

Thiamin is a substituted pyrimidine linked through a methylene bridge ($-CH_2-$) to a substituted thiazole (Figure 7.19) [82]. Thiamin is widely distributed in plant and animal tissues. Most naturally occurring thiamin exists as thiamin pyrophosphate (Figure 7.19), with lesser amounts of nonphosphorylated thiamin, thiamin monophosphate and thiamin triphosphate. Thiamin pyrophosphate functions as a coenzyme of various α-keto acid dehydrogenases, α-keto acid decarboxylases, phosphoketolases, and transketolases. Thiamin is commercially available as the hydrochloride and mononitrate salts, and these forms are widely used for food fortification and as nutritional supplements (Figure 7.19).

The thiamin molecule exhibits unusual acid–base behavior. The first pK_a (~4.8) involves dissociation of the protonated pyrimidine N^1 to yield the uncharged pyrimidyl moiety of thiamin free base (Figure 7.20). In the alkaline pH range another transition is observed (apparent pK_a 9.2) that corresponds to the uptake of two equivalents of base to yield the thiamin pseudobase, followed by opening of the thiazole ring to yield the thiol form of thiamin (Figure 7.20). Another characteristic of the thiamin free base is the quaternary N of the thiazole ring, which remains cationic at all pH values. The marked pH dependence of thiamin degradation corresponds to the pH-dependent changes in ionic and structural forms. Protonated thiamin is far more stable than free base, pseudobase, and thiol forms, which accounts for the greater stability observed in acidic media (Table 7.15) [104]. Although thiamin is relatively stable to oxidation and light, it is among the least stable of the vitamins when in solution at neutral or alkaline pH.

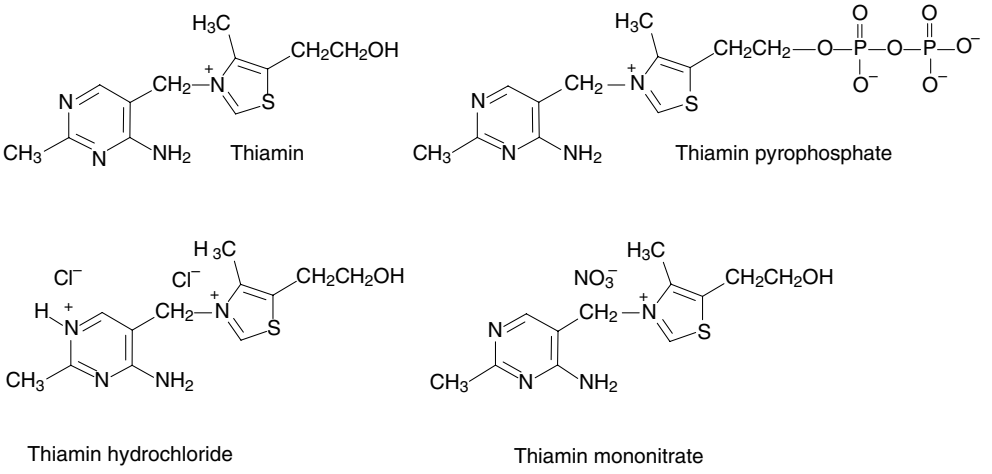


FIGURE 7.19 Structures of various forms of thiamin. All have thiamin (vitamin B_1) activity.

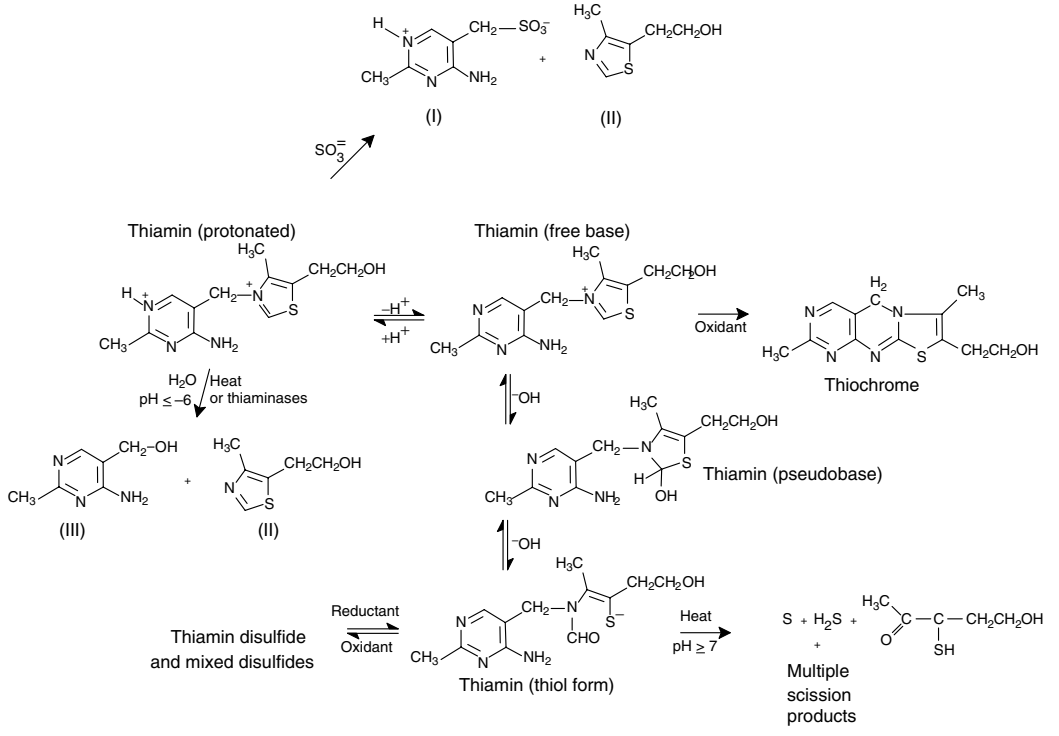


FIGURE 7.20 Summary of the major pathways for ionization and degradation of thiamin. (Adapted in modified form from Tannenbaum, S. R., et al. (1985). in *Food Chemistry, 2nd Edition, Revised and Expanded* (O. R. Fennema, ed.), Marcell Dekker, New York, pp. 477–544; Dwivedi, B. K. and R. G. Arnold. (1973). *J. Agric. Food Chem.* 21:54–60; and Charmichael, E. C., et al. (1997) *J. Chem. Soc. Perkin Trans*. 2:2609–2619.)

TABLE 7.15
Comparison of Thermal Stability of Thiamin and Thiamin Pyrophosphate in 0.1 M Phosphate Buffer at 265°C

	Thiamin		Thiamin Pyrophosphate	
Solution pH	k^a (min^{-1})	$t^{1/2}$ (min)	k^a (min^{-1})	$t^{1/2}$ (min)
4.5	0.0230	30.1	0.0260	26.6
5.0	0.0215	32.2	0.0236	29.4
5.5	0.0214	32.4	0.0358	19.4
6.0	0.0303	22.9	0.0831	8.33
6.5	0.0640	10.8	0.1985	3.49

[a] k is the first-order rate constant and $t^{1/2}$ is the time for 50% thermal degradation.

Source: Adapted from Mulley, E. A., et al. (1975). *J. Food Sci*. 40:989–992.

7.8.2.2 Stability and Modes of Degradation

7.8.2.2.1 Stability Properties

A wealth of published data exists concerning the stability of thiamin in foods [40,98]. Representative studies, as summarized previously by Tannenbaum et al. [140], illustrate the potential for large losses under certain conditions (Table 7.16) [98]. Losses of thiamin from foods are favored when: (1) conditions favor leaching of the vitamin into surrounding aqueous media; (2) the pH is approximately neutral or greater; and (3) exposure to a sulfiting agent occurs. Losses of thiamin also can occur in fully hydrated foods during storage at moderate temperatures, although at predictably lower rates than those observed during thermal processing (Table 7.17) [44]. Thiamin degradation in foods almost always follows first-order kinetics. Because degradation can occur by several possible mechanisms, multiple mechanisms sometimes occur simultaneously. The occurrence of nonlinear Arrhenius plots for thermal losses of thiamin in certain foods is an evidence of multiple degradation mechanisms that have different temperature dependence.

Thiamin exhibits excellent stability under conditions of low water activity at ambient temperature. Thiamin in dehydrated model systems simulating breakfast cereals exhibits little or no loss at temperatures less than 37°C at a_w 0.1–0.65 (Figure 7.21) [19a,36,140]. In contrast, it degrades faster at 45°C, especially at a_w 0.4 or greater (i.e., above the apparent monomolecular moisture value of $a_w \sim 0.24$). In these model systems, the maximum rate of thiamin degradation occurred at water activities of 0.5–0.65 (Figure 7.22) [30]. In similar model systems, the rate of thiamin degradation declined as the a_w was increased from 0.65 to 0.85 [5].

Thiamin is somewhat unstable in many fish and crustaceans postharvest, and this has been attributed to the presence of thiaminases. However, at least part of this thiamin-degradation activity is caused by heme proteins (myoglobin and hemoglobin) that act as nonenzymatic catalysts [113]. The presence of thiamin-degrading heme proteins in tuna, pork, and beef muscle suggests that denatured myoglobin may be involved in the degradation of thiamin during food processing and storage. This nonenzymatic, thiamin-modifying activity apparently does not cause cleavage of the thiamin molecule, as is common in thiamin degradation. The antithiamin component of fish viscera, previously reported to be a thiaminase, is now believed to be a thermostable and probably nonenzymatic catalyst.

Other components of food can influence degradation of thiamin in foods. Tannins can inactivate thiamin apparently by the formation of several biologically inactive adducts. Various flavonoids may alter the thiamin molecule, but the apparent product of flavonoid oxidation in the presence of thiamin is thiamin disulfide, a compound that has thiamin activity. Proteins and carbohydrates can reduce

TABLE 7.16
Representative Rates of Degradation (Half-Life at Reference Temperature of 100°C) and Energy of Activation for Losses of Thiamin from Foods During Thermal Processing

Food System	pH	Temperature Range Studied (°C)	Half-Life (h)	Energy of Activation (KJ/mol)
Beef heart puree	6.10	109–149	4	120
Beef liver puree	6.18	109–149	4	120
Lamb puree	6.18	109–149	4	120
Pork puree	6.18	109–149	5	110
Ground meat product	Not reported	109–149	4	110
Beef puree	Not reported	70–98	9	110
Whole milk	Not reported	121–138	5	110
Carrot puree	6.13	120–150	6	120
Green bean puree	5.83	109–149	6	120
Pea puree	6.75	109–149	6	120
Spinach puree	6.70	109–149	4	120
Pea puree	Not reported	121–138	9	110
Peas in brine puree	Not reported	121–138	8	110
Peas in brine	Not reported	104–133	6	84

Water activity estimated to be 0.98–0.99. Half-life and energy of activation values rounded to 1 and 2 significant figures, respectively.

Source: Mauri, L. M., et al. (1989). *Int. J. Food Sci. Technol.* 24:1–9. Data compiled from multiple sources.

TABLE 7.17
Typical Losses of Thiamin in Canned Foods During Storage

	Retention After 12 Months Storage (%)	
Food	**38°C**	**1.5°C**
Apricots	35	72
Green beans	8	76
Lima beans	48	92
Tomato juice	60	100
Peas	68	100
Orange juice	78	100

Source: Freed, M., et al. (1948). *Food Technol.* 3:148–151.

the rate of thiamin degradation during heating or in the presence of bisulfite, although the extent of this effect is difficult to predict in complex food systems. A part of the stabilizing effect of protein may occur through the formation of mixed disulfides with the thiol form of thiamin, a reaction that appears to retard further modes of degradation. Chlorine (as hypochlorite ion), at levels present in

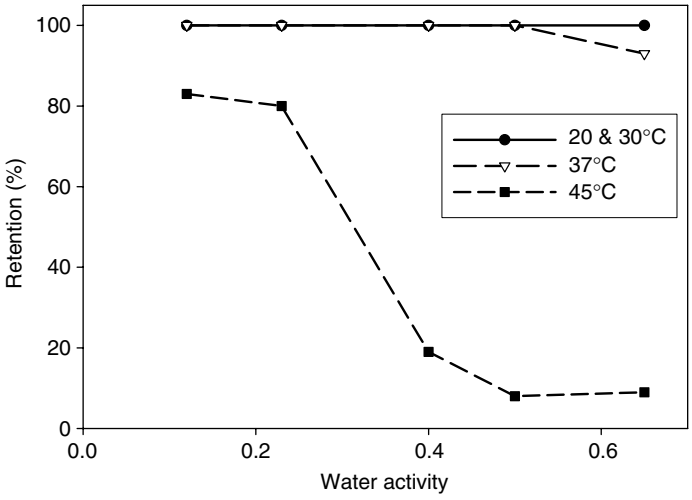


FIGURE 7.21 Influence of water activity and temperature on the retention of thiamin in a dehydrated model food system simulating a breakfast cereal product. Percentage retention values apply to an 8-mo storage period. (From Dennison, D., et al. (1977). *J. Food Process. Preserv.* 1: 43–54.)

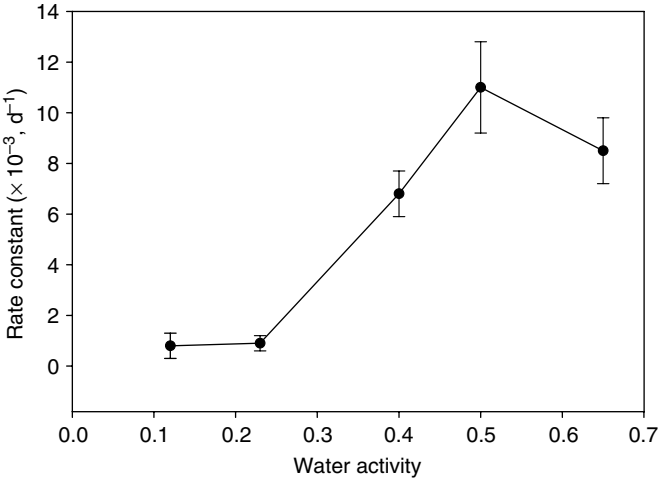


FIGURE 7.22 Influence of water activity on the first-order rate constant of thiamin degradation in a dehydrated model food system simulating a breakfast cereal product stored at 45°C. (From Dennison, D. B., et al. (1977). *J. Food Process. Preserv.* 1: 43–54.)

water used in food formulation and processing, can cause rapid degradation of thiamin by a cleavage process that is apparently identical to the thermal cleavage of thiamin under acidic conditions.

Another complicating factor in the assessment and prediction of thiamin stability is the inherent difference in stability and pH dependence between free thiamin and the major naturally occurring form, thiamin pyrophosphate. Although thiamin and thiamin pyrophosphate exhibit nearly equivalent rates of thermal degradation at pH 4.5, thiamin pyrophosphate degrades almost three times faster at pH 6.5 (Table 7.15).

Significant differences exist in the stability of hydrochloride and mononitrate forms of synthetic thiamin. Thiamin HCl is more soluble than the mononitrate, and this is advantageous for fortification

TABLE 7.18
Kinetic Values for Thiamin Loss in Semolina Dough Subjected to High Temperatures

a_w	Temperature (°C)	k ($\times 10^4$ min^{-1}) ± 95% CI[a]	Half-Life (min)	Energy of Activation (kcal/mol)
Hydrochloride				
0.58	75	3.72 ± 0.01	1863	95.4
	85	11.41 ± 3.64	607	
	95	22.45 ± 2.57	309	
0.86	75	5.35 ± 2.57	1295	92.1
	85	12.20 ± 4.45	568	
	95	30.45 ± 8.91	228	
Mononitrate				
0.58	75	2.88 ± 0.01	2406	109
	85	7.91 ± 0.01	876	
	95	22.69 ± 2.57	305	
0.86	75	2.94 ± 0.01	2357	111
	85	8.31 ± 0.01	834	
	95	23.89 ± 0.01	290	

[a] First-order rate constant ± 95% confidence interval (CI).

Source: Labuza, T. P. and J. F. Kamman (1982). *J. Food Sci.* 47:664–665.

of liquid products. Because of differing energies of activation, thiamin mononitrate is more stable at temperatures less than 95°C, while the hydrochloride exhibits greater stability at temperatures >95–110°C (Table 7.18) [87].

7.8.2.2.2 Mechanisms of Degradation

The rate and mechanism of thermal degradation of thiamin are strongly influenced by pH of the reaction medium, but degradation usually involves cleavage of the molecule at the central methylene bridge.

In acidic conditions (i.e., pH $\leq$ 6), thermal degradation of thiamin occurs slowly and involves cleavage of the methylene bridge to release the pyrimidine and thiazole moieties largely in unchanged form. Between pH 6 and 7, thiamin degradation accelerates along with a large increase in the extent of fragmentation of the thiazole ring, and at pH 8 intact thiazole rings are not found among the products. Thiamin degradation is known to yield a large number of sulfur-containing compounds that presumably arise from fragmentation and rearrangement of the thiazole ring. These compounds have been shown to contribute to meat flavor. Products from thiazole fragmentation are thought to arise from the small amounts of thiamin that exist in the thiol or pseudobase forms at pH > 6.

Thiamin degrades rapidly in the presence of bisulfite ions, a phenomenon that stimulated federal regulations prohibiting the use of sulfiting agents in foods that are significant sources of dietary thiamin. The cleavage of thiamin by bisulfite is similar to that occurring at pH $\leq$ 6, although the pyrimidine product is sulfonated (Figure 7.20). This reaction is described as a base exchange or nucleophilic displacement at the methylene carbon, by which the bisulfite ion displaces the thiazole moiety. It is unclear whether other nucleophiles relevant to foods can have a similar effect. Cleavage of thiamin by bisulfite occurs over a broad pH range, with a maximum rate occurring at pH $\sim$ 6 [161]. A bell-shaped pH profile of this reaction occurs because the sulfite ion primarily reacts with the protonated form of thiamin.

Several researchers have noted a correspondence of the conditions (e.g., pH and water activity) favoring degradation of thiamin and progress of the Maillard reaction. Specifically, thiamin, which

has a primary amino group on its pyrimidyl moiety, shows a maximum rate of degradation at an intermediate water activity and exhibits greatly increased reaction rates at neutral and alkaline pH values. Early studies demonstrated the ability of thiamin to react with sugars under certain conditions; however, sugars often tend to increase the stability of thiamin. Despite the similarity of conditions favoring thiamin degradation and Maillard browning, there appears to be little or no direct interaction of thiamin with the reactants or intermediates of the Maillard reaction in foods.

7.8.2.3 Bioavailability

Although the bioavailability of thiamin has not been fully evaluated, its utilization appears to be nearly complete in most foods examined [52,70]. As mentioned previously, formation of thiamin disulfide and mixed thiamin disulfides during food processing apparently has little effect on thiamin bioavailability. Thiamin disulfide exhibits 90% of the activity of thiamin in animal bioassays.

7.8.2.4 Analytical Methods

Although microbiological growth methods exist for measurement of thiamin in foods, they are rarely used because of the availability of fluorometric and HPLC procedures [41]. Thiamin is generally extracted from the food by heating (e.g., autoclaving) a homogenate in dilute acid. For analysis of total thiamin, treatment of the buffered extract with a phosphatase hydrolyzes phosphorylated forms of the vitamin. Following chromatographic removal of nonthiamin fluorophores, treatment with an oxidizing agent converts thiamin to the highly fluorescent thiochrome that is easily measured (Figure 7.20).

Total thiamin can be determined by HPLC following phosphatase treatment. Fluorometric HPLC analysis can be used following conversion of thiamin to thiochrome or, alternatively, postcolumn oxidation to thiochrome can permit fluorometric detection. Individual phosphate esters of thiamin can be determined simultaneously by HPLC.

7.8.3 Riboflavin

7.8.3.1 Structure and General Properties

Riboflavin, formerly known as vitamin B_2, is the generic term for the group of compounds that exhibit the biological activity of riboflavin (Figure 7.24). The parent compound of the riboflavin family is 7,8-dimethyl-10(1′-ribityl)isoalloxazine, and all derivatives of riboflavin are given the generic name flavins. Phosphorylation of the 5′-position of the ribityl side chain yields flavin mononucleotide (FMN), whereas flavin adenine dinucleotide (FAD) has an additional 5′-adenosyl monophosphate moiety (Figure 7.23) [30]. FMN and FAD function as coenzymes in a large number of flavin-dependent enzymes that catalyze various oxidation–reduction processes. Both forms are readily convertible to riboflavin by action of phosphatases that are present in foods and those of the digestive system. A relatively minor fraction (<10%) of the FAD in biological materials exists in a covalently bound coenzyme form in which position 8α is covalently linked to an amino acid residue of the enzyme protein.

The chemical behavior of riboflavin and other flavins is complex, with each form able to exist in several oxidation states as well as multiple ionic forms [117]. Riboflavin, as the free vitamin and in its coenzymic function, undergoes redox cycling among three chemical species. These include the native (fully oxidized) yellow flavoquinone (Figure 7.24), the flavosemiquinone (red or blue depending on pH), and the colorless flavohydroquinone. Each conversion in this sequence involves a one-electron reduction and H^+ uptake. The flavosemiquinone N^5 has a pK_a of ~8.4, while the flavohydroquinone N^1 has a pK_a of ~6.2.

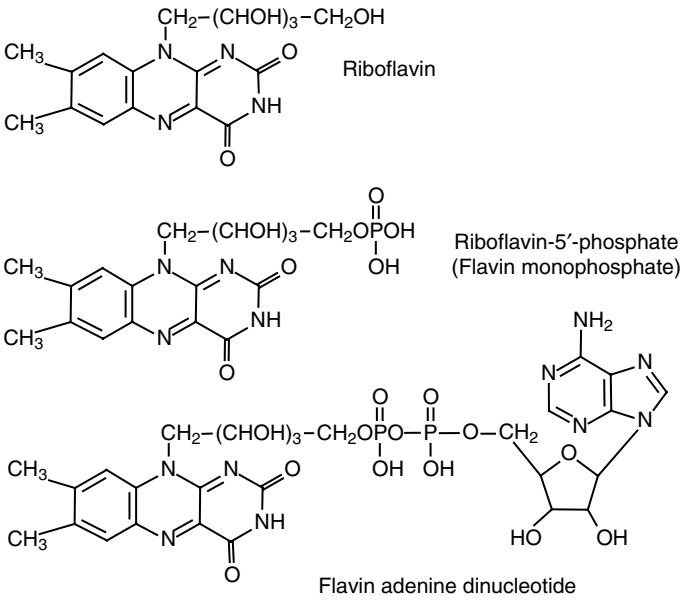


FIGURE 7.23 Structures of riboflavin, flavin mononucleotide and flavin adenine dinucleotide. Positions of isoalloxazine ring system are designated. The asterisk (*) on the 8α carbon of flavin adenine dinucleotide indicates the site of covalent linkage to amino acids (cys or his) in certain enzymes.

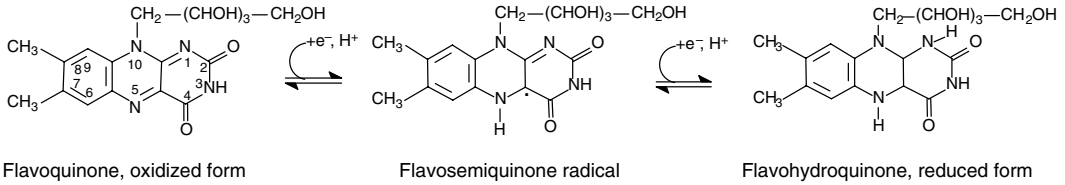


FIGURE 7.24 Oxidation–reduction behavior of flavins.

Several minor forms of riboflavin also exist in foods, although their chemical origin and quantitative significance in human nutrition have not been fully determined. As shown in Table 7.19, FAD and free riboflavin account for over 80% of the total flavins in cow's and human milk [120,121]. Of the minor forms present, most interesting is 10-hydroxyethylflavin, a product of bacterial flavin metabolism. 10-Hydroxyethylflavin is a known inhibitor of mammalian flavokinase and may inhibit the uptake of riboflavin into tissues. Other minor derivatives (such as lumiflavin) may also act as antagonists. Thus, foods contain flavins such as riboflavin, FAD, and FMN, that exhibit vitamin activity, but in addition they may contain compounds that act as antagonists of riboflavin transport and metabolism. This illustrates the need for a thorough analysis of the forms of riboflavin and other vitamins in order to assess accurately the nutritional properties of foods.

7.8.3.2 Stability and Modes of Degradation

Riboflavin exhibits its greatest stability in acidic medium and is somewhat less stable at neutral pH, and rapidly degrades in alkaline environments. Retention of riboflavin in most foods is moderate to very good during conventional thermal processing, handling, and preparation. Losses during storage of riboflavin in various dehydrated food systems (breakfast cereals and model systems) are

TABLE 7.19
Distribution of Riboflavin Compounds in Fresh Human and Cow's Milk

Compound	Human Milk (%)	Cow's Milk (%)
FAD	38–62	23–46[a]
Riboflavin	31–51	35–59
10-Hydroxyethylflavin	2–10	11–19
10-Formylmethyflavin	Trace	Trace
7α-Hydroxyriboflavin	Trace–0.4	0.1–0.7
8α-Hydroxyriboflavin	Trace	Trace–0.4

[a] Following pasteurization, FAD in bulk raw milk decreases from 26% to 13%, with a corresponding increase in the percentage of riboflavin.

Source: Adapted from Roughead, Z. K. and D. B. McCormick (1990). *J. Nutr.* 120:382–388; Roughead, Z. K. and D. B. McCormick. (1990). *Am. J. Clin. Nutr.* 52:854–857.

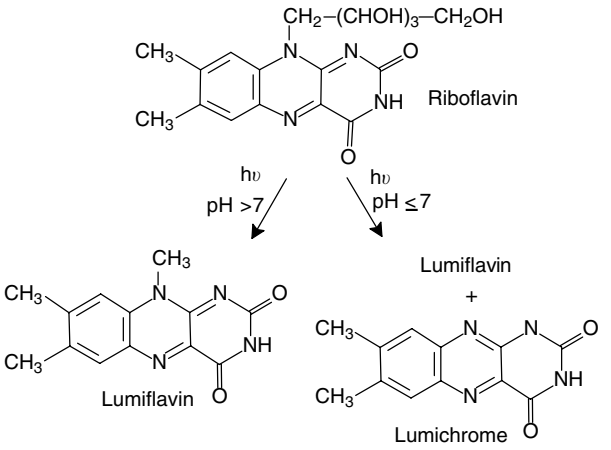


FIGURE 7.25 Photochemical conversion of riboflavin to lumichrome and lumiflavin.

usually negligible. Rates of degradation increase measurably at a_ws above the monolayer value when temperatures are above ambient [30].

The typical mechanism of degradation of riboflavin is photochemical that yields two biologically inactive products lumiflavin and lumichrome (Figure 7.25), and an array of free radicals [155]. Exposure of solutions of riboflavin to visible light has been used for many years as an experimental technique to generate free radicals. Photolysis of riboflavin yields superoxide and riboflavin radicals ($R^{\bullet}$), and the reaction of O_2 with $R^{\bullet}$ provides peroxy radicals and a wide range of other products. The extent to which photochemical degradation of riboflavin is responsible for photosensitized oxidation reactions in foods has not been quantitatively determined, although this process assuredly contributes significantly. Riboflavin is involved in the photosensitized degradation of AA and presumably other labile vitamins. Light-induced off-flavor in milk, which is mediated by exposure to sunlight or fluorescent light, is a riboflavin-mediated photochemical process. Although the mechanism of off-flavor formation has not been fully determined, light-induced (probably radical-mediated)

decarboxylation and deamination of methionine to form methional ($CH_3-S-CH_2-CH_2-CH=O$) is at least partially responsible. Concurrent mild oxidation of milk lipids also occurs. The incidence of light-induced off-flavor has been reduced by the demise of home delivery of milk in glass bottles and changes in packaging materials.

7.8.3.3 Bioavailability

Relatively little is known regarding the bioavailability of naturally occurring forms of riboflavin; however, there is little evidence of problems associated with incomplete bioavailability. The covalently bound forms of FAD coenzymes have been shown to exhibit very low availability when administered to rats, although these are minor forms of the vitamin. The nutritional significance of dietary riboflavin derivatives that have potential antivitamin activity has not yet been determined in animals or humans.

7.8.3.4 Analytical Methods

Flavins are highly fluorescent compounds in their fully oxidized flavoquinone form (Figure 7.24), and this property serves as the basis for most analytical methods. The traditional assay procedure for the measurement of total riboflavin in foods involves measurement of fluorescence before and after chemical reduction to the nonfluorescent flavohydroquinone [130]. Fluorescence is a linear function of concentration in dilute solution, although certain food components can interfere with accurate measurement. Several HPLC methods are also suitable for measurement of total riboflavin in food extracts [41,147]. These HPLC procedures and the fluorometric method require extraction by autoclaving in dilute acid followed by a phosphatase treatment to release riboflavin from FMN and FAD. HPLC can also be used to measure the individual riboflavin compounds in foods [120].

7.8.4 Niacin

7.8.4.1 Structure and General Properties

Niacin is the generic term for pyridine 3-carboxylic acid (nicotinic acid) and derivatives that exhibit similar vitamin activity (Figure 7.26). Nicotinic acid and the corresponding amide (nicotinamide;

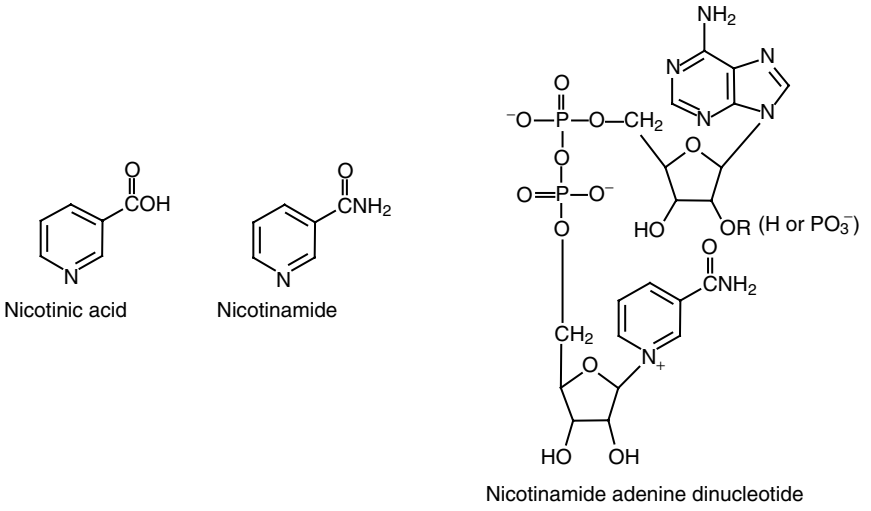


FIGURE 7.26 Structures of nicotinic acid, nicotinamide, and nicotinamide adenine dinucleotide (phosphate). In NAD and NADP, reduction occurs by acceptance of a hydride unit at the C-4 position of the pyridine ring.

pyridine 3-carboxamide) are probably the most stable of the vitamins. The coenzyme forms of niacin are nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), either of which can exist in oxidized or reduced form. NAD and NADP function as coenzymes (in the transfer of reducing equivalents) in many dehydrogenase reactions. Heat, especially under acid or alkaline conditions, converts nicotinamide to nicotinic acid without loss of vitamin activity. Niacin is not affected by light, and no thermal losses occur under conditions relevant to food processing. As with other water-soluble nutrients, losses can occur by leaching during washing, blanching, and processing/preparation and by exudation of fluids from tissues (i.e., drip). Niacin is widely distributed in vegetables and foods of animal origin. Niacin deficiency is rare in the United States partially as a result of programs to enrich cereal grain products with this nutrient. Diets high in protein reduce the requirement for dietary niacin because of the metabolic conversion of tryptophan to nicotinamide.

In certain cereal grain products, niacin exists in several chemical forms that, unless hydrolyzed, exhibit no niacin activity. These inactive niacin forms include poorly characterized complexes involving carbohydrates, peptides, and phenols. Analysis of these nutritionally unavailable, chemically bound forms of niacin has revealed chromatographic heterogeneity and variation in chemical composition, indicating that many bound forms of niacin exist naturally. Alkaline treatments release niacin from these complex derivatives, which permit measurement of total niacin. Several esterified forms of nicotinic acid exist naturally in cereal grains, and these compounds contribute little to niacin activity in foods.

Trigonelline, or *N*-methyl-nicotinic acid, is a naturally occurring alkaloid found at relatively high concentrations in coffee and at lower concentrations in cereal grains and legumes. Under the mildly acidic conditions that prevail during roasting of coffee, trigonelline is demethylated to form nicotinic acid, yielding a 30-fold increase in the niacin concentration and activity of coffee.

Cooking also changes the relative concentration of certain niacin compounds through interconversion reactions [150,151]. For example, heating releases free nicotinamide from NAD and NADP during the boiling of corn. In addition, the distribution of niacin compounds within a product varies as a function of variety (e.g., sweet corn vs. field corn) and stage of maturity.

7.8.4.2 Bioavailability

The existence of nutritionally unavailable forms of niacin in many foods of plant origin has been known for many years, although the chemical identities of the unavailable forms of the vitamin are poorly characterized. In addition to the chemically bound forms discussed above, several other forms of niacin contribute to its incomplete availability in foods of plant origin [150]. NADH, the reduced form of NAD, and presumably NADPH, exhibit very low bioavailability because of their instability in the gastric acid environment. This may be of little nutritional significance because of the low concentration of these reduced forms in many foods. The primary factor affecting niacin bioavailability is the proportion of the total niacin that is chemically bound. As shown in Table 7.20 [150], there is often much more niacin measurable following alkaline extraction than there is by rat bioassays (biologically available niacin) or by direct analysis (free niacin).

7.8.4.3 Analytical Methods

Niacin can be measured by microbiological assay. The principal chemical assay involves a reaction of niacin with cyanogen bromide to yield an N-substituted pyridine that is then coupled to an aromatic amine to form a chromophore [37]. Several HPLC methods are available for measurement of nicotinic acid and nicotinamide in foods [41,147], and HPLC has been used to determine individual free and bound forms of niacin in cereal grains [150,151].

TABLE 7.20
Concentration of Niacin in Selected Foods as Determined by Chemical Assay (Acidic or Alkaline Extraction Methods) or Rat Bioassay. Analysis of HCl Extract Yields a Measure of "Free Niacin," Assay of the Alkaline Extract Provides a Measure of Total Niacin, and the Rat Bioassay is a Measure of Biologically Available Niacin

	Type of Chemical Assay		
Food	**Free Niacin (μg/g)[a]**	**Total Niacin (Alkaline Extraction) (μg/g)[a]**	**Rat Bioassay (μg/g)[a]**
Corn	0.4	25.7	0.4
Boiled corn	3.8	23.8	6.8
Corn after alkaline heating (liquid retained)	24.6	24.6	22.3
Tortillas	11.7	12.6	14
Sweet corn (raw)	—	54.5	40
Steamed sweet corn	45	56.4	48
Boiled sorghum grain	1.1	45.5	16
Boiled rice	17	70.7	29
Boiled wheat	—	57.3	18
Baked potatoes	12	51	32
Baked liver	297	306	321
Baked beans	19	24	28

[a] Wet weight basis.

Source: Adapted from Wall, J. S. and K. J. Carpenter. (1988). *Food Technol*. 42:198–204.

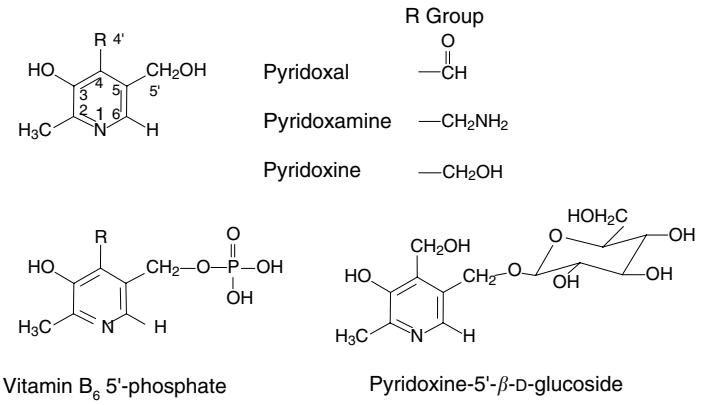


FIGURE 7.27 Structures of vitamin B_6 compounds.

7.8.5 Vitamin B_6

7.8.5.1 Structure and General Properties

Vitamin B_6 is a generic term for the group of 2-methyl, 3-hydroxy, 5-hydroxymethyl-pyridines having the vitamin activity of pyridoxine (PN). The various forms of vitamin B_6 differ according to the nature of the one-carbon substituent at the 4-position, as shown in Figure 7.27. For PN the

TABLE 7.21
pK_a Values of Vitamin B_6 Compounds

	pK_a				
Ionization[a]	**PN**	**PL**	**PM**	**PLP**	**PMP**
3-OH	5.00	4.20–4.23	3.31–3.54	4.14	3.25–3.69
Pyridinium N	8.96–8.97	8.66–8.70	7.90–8.21	8.69	8.61
4′-Amino group			10.4–10.63		ND
5′-Phosphate ester					
pK_{a1}				<2.5	<2.5
pK_{a2}				6.20	5.76

[a] Abbreviations: PN, pyridixine; PL, pyridoxal; PM, pyridoxamine; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate. ND, not determined.

Source: Snell, E. E. (1963). *Compr. Biochem.* 2: 48–58.

substituent is an alcohol, for pyridoxal (PL) it is an aldehyde, and for pyridoxamine (PM) it is an amine. These three basic forms can also be phosphorylated at the 5′-hydroxymethyl group, yielding pyridoxine 5′-phosphate (PNP), pyridoxal 5′-phosphate (PLP), or pyridoxamine 5′-phosphate (PMP). Vitamin B_6, in the form of PLP and to a lesser extent, PMP, functions as a coenzyme in over 100 enzymatic reactions involved in the metabolism of amino acids, carbohydrates, neurotransmitters, and lipids. All of the mentioned forms of vitamin B_6 possess vitamin activity because they can be converted *in vivo* to these coenzymes. The use of "pyridoxine" as a generic term for vitamin B_6 has been discontinued. Similarly, the term "pyridoxol" has been discontinued in favor of "pyridoxine."

Glycosylated forms of vitamin B_6 are present in most fruits, vegetables, and cereal grains, generally as pyridoxine-5′-β-D-glucoside (Figure 7.27; [56,157]). These comprise 5–75% of the total vitamin B_6 and account for 15–20% of the vitamin B_6 in typical mixed diets. Pyridoxine glucoside becomes nutritionally active only after hydrolysis of the glucoside by the action of β-glucosidases in the intestine or other organs. Several other glycosylated forms of vitamin B_6 are also found in certain plant products.

Vitamin B_6 compounds exhibit complex ionization that involves several ionic sites (Table 7.21) [134]. Because of the basic character of the pyridinium N (p$K_a \sim 8$) and the acidic nature of the 3-OH (p$K_a \sim 3.5$–5.0), the pyridine ring system of vitamin B_6 molecules mainly exists in zwitterionic form at neutral pH. The net charge on vitamin B_6 compounds varies markedly as a function of pH. The 4′-amino group of PM and PMP (p$K_a \sim 10.5$), and the 5′-phosphate ester of PLP and PMP (p$K_a < 2.5$, ~6, and ~12) also contribute to the charge of these forms of the vitamin.

All chemical forms of vitamin B_6 exist in foods, although the distribution varies markedly. PN-glucoside exists only in plant products, although most plant products also contain all other forms of the vitamin. Vitamin B_6 in muscle and organ meats is predominantly (>80%) PLP and PMP, with minor amounts of the nonphosphorylated species. Disruption of raw plant tissues by freeze-thaw cycling or homogenization releases phosphatases and β-glucosidases that can alter the forms of vitamin B_6 compounds by catalyzing dephosphorylation and deglycosylation reactions. Similarly, disruption of animal tissues prior to cooking can cause extensive dephosphorylation of PLP and PMP. PNP is a transient intermediate in vitamin B_6 metabolism and is usually a negligible component of the total vitamin B_6 content. PN (as the HCl salt) is the form of vitamin B_6 used for food fortification and in nutritional supplements because of its excellent stability. Intakes of supplemental vitamin B_6 above 100 mg/d should be avoided because of its potential neurotoxicity. Supplements containing any other form of vitamin B_6, such as pyridoxal phosphate, offer no added

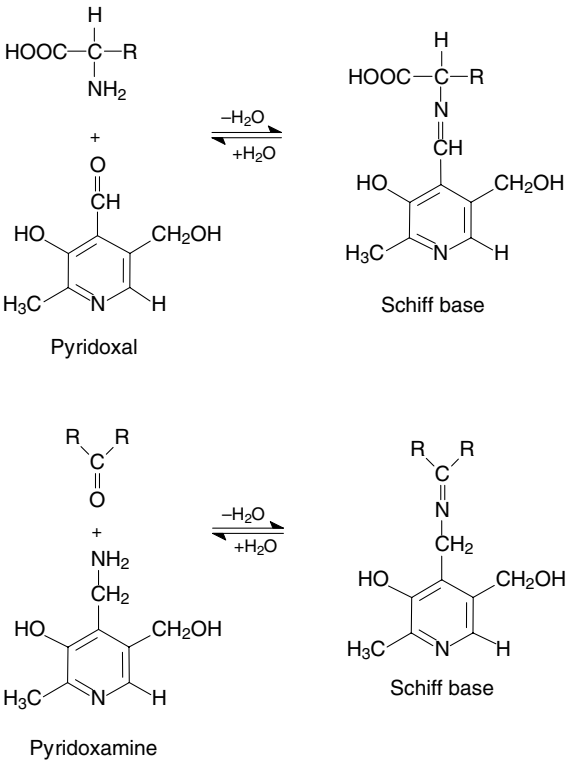


FIGURE 7.28 Formation of Schiff base structures from pyridoxal (PL) and pyridoxamine (PM). Analogous reactions occur with PLP and PMP.

nutritional benefit beyond that of PN. Aside from the HCl form, PN has been marketed in the form of the pyridoxine-α-ketoglutarate (PAK) complex. The claims regarding PAK are poorly substantiated, and the doses reported (typically >100 mg PN equivalents/d) may be hazardous.

The aldehyde and amine forms of vitamin B_6 readily participate in carbonyl-amine reactions: PLP or PL with amines, or PMP or PM with aldehydes or ketones (Figure 7.28). The coenzymic action of PLP in most B_6-dependent enzymes involves a carbonyl-amine condensation. PLP and PL readily form Schiff bases with the neutral amino groups of amino acids, peptides, or proteins. Coordinate covalent bonding to a metal ion increases the stability of the Schiff base in nonenzymatic systems, although Schiff bases can exist in solutions devoid of metal ions. PLP forms Schiff bases much more readily than PL because the phosphate group of PLP blocks the formation of an internal hemiacetal and maintains the carbonyl in reactive form (Figure 7.29; 154). As with other carbonyl-amine reactions, the nonenzymatic formation of a vitamin B_6 Schiff base is strongly pH dependent and exhibits an alkaline pH optimum. The stability of the Schiff base complexes is also strongly pH dependent, with dissociation occurring in acidic environments. Thus, Schiff-base forms of vitamin B_6 would be expected to dissociate fully in acidic media such as the postprandial gastric contents. In addition to the Schiff base in Figure 7.28, several other tautomeric and ionic forms exist in an equilibrium situation.

Depending on the chemical nature of the amino compound condensing with PLP or PL in the Schiff base, further rearrangement to various cyclic structures can occur. For example, cysteine condenses with PL or PLP to form a Schiff-base, then the SH group attacks the Schiff base 4′-C to form the cyclic thiazolidine derivative (Figure 7.30). Histidine, tryptophan, and several related compounds (e.g., histamine and tryptamine) can form similar cyclic complexes with PL or PLP bases through reactions of the imidazolium and indolyl side chains, respectively. These cyclic derivatives

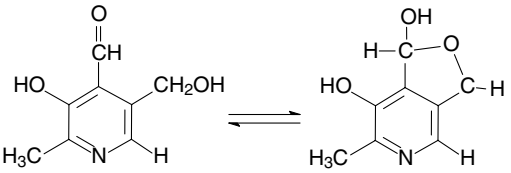


FIGURE 7.29 Formation of pyridoxal hemiacetal.

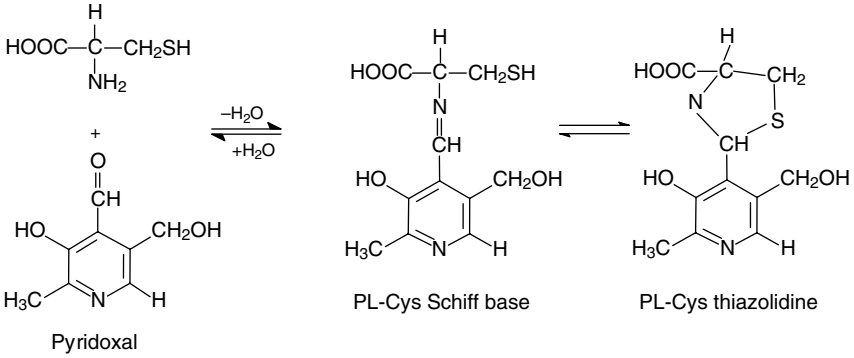


FIGURE 7.30 Formation of the Schiff base and thiazolidine complexes of pyridoxal (PL) and cysteine.

are believed to have full bioavailability because the gastric pH presumably would promote the dissociation of the PL or PLP from the amino acid.

7.8.5.2 Stability and Modes of Degradation

Thermal processing and storage of foods can influence the vitamin B_6 content in several ways. As with other water-soluble vitamins, exposure to water can cause leaching and consequent losses. Chemical changes can involve interconversion of chemical forms of vitamin B_6, thermal or photochemical degradation, as well as irreversible complexation with proteins, peptides, or amino acids.

The interconversion of vitamin B_6 compounds occurs mainly by nonenzymatic transamination, which involves formation of a Schiff base and migration of the Schiff base double bond, followed by hydrolysis and dissociation. Nonenzymatic transamination has been studied extensively as a model of PLP-mediated enzymatic transaminations. This process occurs extensively during the thermal processing of foods that contain the aldehyde or amine forms of vitamin B_6. For example, increases in the proportion of PM and PMP are frequently observed in the cooking or thermal processing of meat and dairy products [11,57] and in studies of protein-based liquid model systems [55]. The occurrence of such transamination has no adverse nutritional effect. Similar transamination has been shown to occur during the storage of intermediate moisture, model food systems ($a_w \sim 0.6$). PL-mediated nonenzymatic generation of H_2S and methyl mercaptan from sulfur-containing amino acids can also occur during food processing. This can be a significant source of flavor and can cause discoloration in canned foods through formation of black FeS [58].

All vitamin B_6 compounds are susceptible to light-induced degradation, which can cause losses during food processing, preparation, and storage, and during analysis. The mechanism of vitamin B_6 degradation by light is not well understood and the relationship between reaction rate and wavelength is not known. Light-mediated oxidation appears to be involved, presumably with a free radical intermediate. Exposure of vitamin B_6 to light causes formation of the nutritionally inactive derivatives 4-pyridoxic acid (from PL and PM) and 4-pyridoxic acid 5′-phosphate (from PLP and PMP), providing evidence of susceptibility to photochemical oxidation [116,124]. However, the rates of

TABLE 7.22
Influence of Temperature, Water Activity, and Light Intensity on Degradation of Pyridoxal in a Dehydrated Model Food System

Light Intensity (lumens/m^2)	a_w	Temperature (°C)	k^a (day^{-1})	$t^{1/2^a}$ (days)
4300	0.32	5	0.092	7.4
		28	0.1085	6.4
		37	0.2144	3.2
		55	0.3284	2.1
4300	0.44	5	0.0880	7.9
		28	0.1044	6.6
		55	0.3453	2.0
2150	0.32	27	0.0675	10.3

[a] First-order rate constant and time for 50% degradation, respectively.

Source: Adapted from Saidi, B. and J. J. Warthesen (1983). *J. Agric. Food Chem*. 31:876–880.

photochemical degradation of PLP, PMP, and PM, and the amount of degradation products obtained, differ only slightly in the presence or absence of air, suggesting that initiation of oxidation does not require direct attack by O_2. Photochemical degradation of PL in low-moisture model food systems occurs at a greater rate than that of PL and PN. The reactions are first order in PL concentration, are strongly influenced by temperature, but are affected only slightly by water activity (Table 7.22) [124].

The rate of nonphotochemical degradation of vitamin B_6 is strongly dependent on the form of the vitamin, temperature, pH of the solution, and the presence of other reactive compounds (e.g., proteins, amino acids, and reducing sugars). All forms of vitamin B_6 exhibit excellent stability at low pH (e.g., 0.1 M HCl), a condition used during traditional extraction methods for vitamin B_6 analysis. During incubation of vitamin B_6 compounds at 40 or 60°C in aqueous solutions buffered at pH 4–7 for up to 140 days, PN exhibited no loss, PM exhibited the greatest loss at pH 7, and PL showed its greatest loss at pH 5 (Table 7.23) [124]. Degradation of PL and PM followed first-order kinetics in these studies. In contrast, similar studies of the degradation of PL, PN, and PM at 110–145°C in aqueous solution buffered at pH 7.2, these compounds exhibited kinetics best described as second order, 1.5 order, and pseudo-first order, respectively [106]. In parallel studies, thermal degradation of vitamin B_6 in cauliflower puree also did not conform to first-order kinetics. The reasons responsible for the kinetic differences of these studies are not clear. Under conditions of dry heat simulating toasting processes, degradation of PN in dehydrated model systems exhibited consistent first-order kinetics [39].

Studies of the thermal stability of vitamin B_6 in foods are complicated because the multiple forms of the vitamin can undergo various degradation reactions, and interconversions also can occur among the various forms of the vitamin. Losses of total vitamin B_6 in food processing or storage are similar to those observed with other water-soluble vitamins. For example, garbanzo and lima beans exhibit ~20–25% loss of total vitamin B_6 during blanching and canning process.

The development of HPLC methods has facilitated studies of the chemical behavior of vitamin B_6 compounds during processing and storage. The simultaneous interconversion of PL and PM, along with first-order loss of total vitamin B_6, has been observed in studies of thermal processing and storage of intermediate moisture model food systems and in liquid model systems simulating infant formula (Figure 7.31; Table 7.24) [55]. PN exhibited greater stability than PL or PM, although the magnitude of the difference varied with temperature (Table 7.23). Although differences in energy of activation suggest a difference in degradation mechanism for these three forms of the vitamin,

TABLE 7.23
Influence of pH and Temperature on the Degradation of Pyridoxal and Pyridoxamine in Aqueous Solution. No Significant Degradation of Pyridoxine was Found at pH 4–7 at 40 or 60°C for up to 140 Days

Compound	Temperature (°C)	pH	k^a (day^{-1})	$t^{1/2^a}$ (days)
Pyridoxal	40	4	0.0002	3466
		5	0.0017	407
		6	0.0011	630
		7	0.0009	770
Pyridoxal	60	4	0.0011	630
		5	0.0225	31
		6	0.0047	147
		7	0.0044	157
Pyridoxamine	40	4	0.0017	467
		5	0.0024	289
		6	0.0063	110
		7	0.0042	165
Pyridoxamine	60	4	0.0021	330
		5	0.0044	157
		6	0.0110	63
		7	0.0108	64

[a] First-order rate constant and time for 50% degradation, respectively.

Source: Adapted from Saidi, B. and J. J. Warthesen (1983). *J. Agric. Food Chem.* 31:876–880.

thermodynamic calculations provide evidence of a common rate- limiting step for loss of PL, PM, and PN [50]. There is a need for further studies to assess more fully the behavior of naturally occurring vitamin B_6 in various foods.

Extensive examination of the thermal stability of vitamin B_6 in milk products was prompted by an unfortunate incident involving infant formulas. In the early 1950s, over 50 cases of convulsive seizures occurred in infants that had consumed a commercially available milk-based infant formula [23], whereas thousands of infants consumed the same formula without ill effects. These convulsive disorders were corrected by administration of PN to the infants. The problem of inadequate vitamin B_6 content in the processed formulas was corrected by fortification with PN, which is much more stable than PL, the major naturally occurring form of the vitamin in milk [64]. Commercial sterilization of evaporated milk or unfortified infant formula causes 40–60% loss of the naturally occurring vitamin B_6. Little or no loss of added PN was found during a comparable thermal process. This incident highlights the need for complete and thorough assessment of the nutritional quality of foods, especially when new formulations and processing methods are employed.

The occurrence of vitamin B_6 deficiency in the case of these unfortified infant formulas has been attributed, at least in part, to the interaction of PL with milk proteins during processing to form a sulfur-containing derivative, *bis*-4-pyridoxyl-disulfide (Figure 7.32) [55]. *Bis*-4-pyridoxyl-disulfide has been reported to form slowly after heating a concentrated solution of PL and cysteine [153], and it exhibits only partial (~20%) vitamin activity in rat bioassays. Evidence of an involvement of sulfhydryl groups in the interaction of PL with milk proteins also has been observed [135]. However, HPLC analysis of thermal processing of milk containing radiolabeled PL and PLP revealed no evidence of the formation of *bis*-4-pyridoxyl-disulfide [57]. Alternatively, PL and PLP were found to undergo extensive binding to lysyl ε-amino groups of milk proteins by reduction of the Schiff

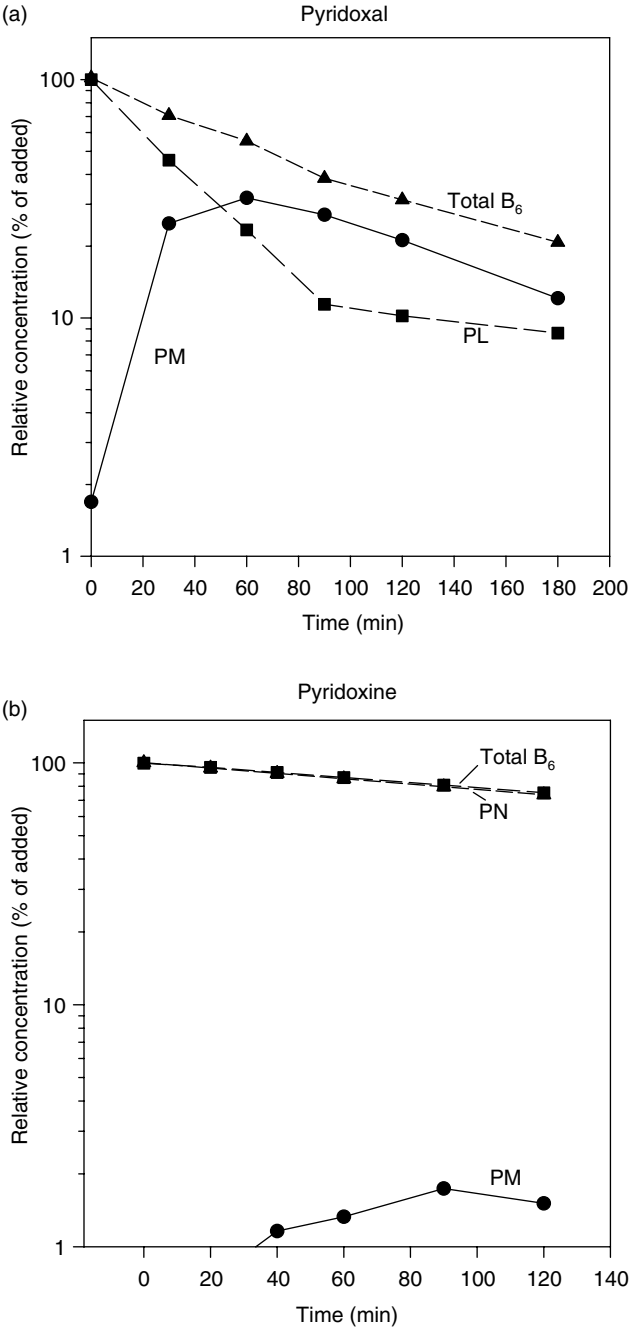


FIGURE 7.31 Degradation and interconversion of vitamin B_6 compounds during thermal processing at 118°C of a liquid model food system simulating infant formula. (from Ref. 55). Original vitamin B_6 content (a) 100% pyridoxal (PL), (b) 100% pyridoxine (PN), and (c) 100% pyridoxamine (PM).

base $-C{=}N-$ linkage (Figure 7.33). The formation of such pyridoxyllysyl residues also has been detected in thermally processed muscle and liver and during storage of intermediate moisture model food systems. The mechanism by which the reduction of the Schiff base linkage occurs has not been determined.

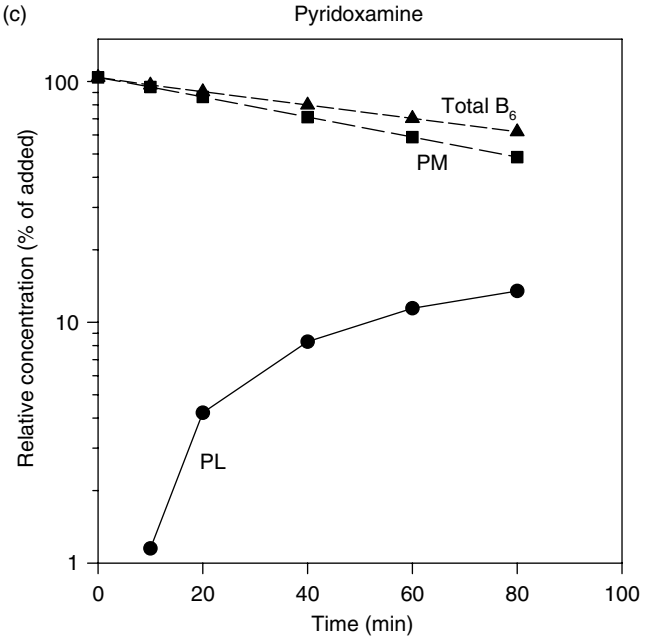


FIGURE 7.31 Continued.

TABLE 7.24
Rate Constants and Energies of Activation for the Thermal Loss of Total Vitamin B_6 in Liquid Model System Simulating Infant Formula

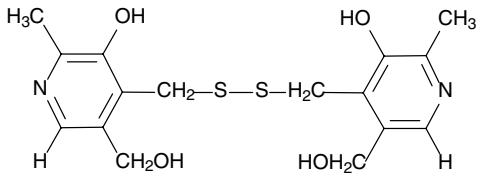
Form of Vitamin B_6 Added	Temperature (°C)	k^a (min^{-1})	$t^{1/2^a}$ (min)	Energy of Activation (KJ/mol)
Pyridoxine	105	0.0006	1120	114
	118	0.0025	289	
	133	0.0083	62	
Pyridoxamine	105	0.0021	340	99.2
	118	0.0064	113	
	133	0.0187	35	
Pyridoxal	105	0.0040	179	87.0
	118	0.0092	75	
	133	0.0266	24	

[a] First-order rate constant and time for 50% degradation, respectively.

Source: Gregory, J. F. and M. E. Hiner. (1983). *J. Food Sci*. 48:1323–1327, 1339.

Pyridoxyllysyl residues associated with food proteins have been shown to exhibit ~50% of the vitamin B_6 activity of PN [47]. When administered to vitamin B_6 deficient rats, this compound exacerbates the deficiency. This effect may have been involved in the deficiencies associated with consumption of an unfortified thermally processed infant formula mentioned earlier.

The role of protein sulfhydryl groups in the interaction of PL with protein has not been fully resolved. Sulfhydryls, as is true of ε-amino or imidazolium groups of amino acid side chains, can



Bis-4-pyridoxyl-disulfide

FIGURE 7.32 Structure of *bis*-4-pyridoxyl-disulfide.

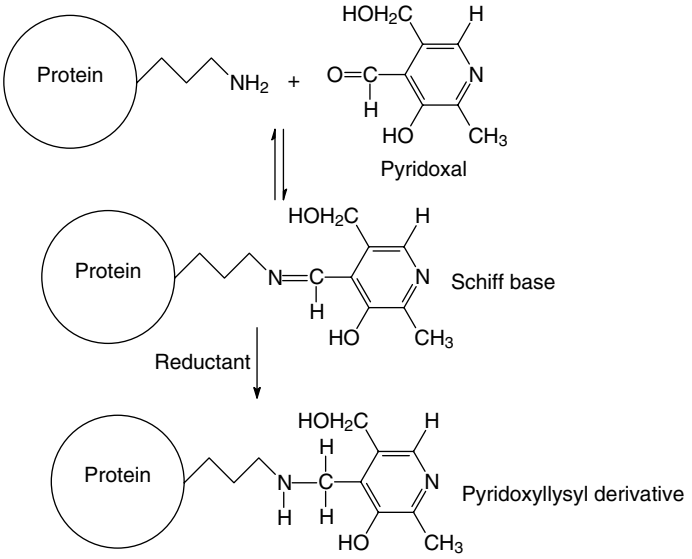


FIGURE 7.33 Interaction of PL with the ε-amino group of a lysyl residue of a food protein to form a Schiff base, followed by reduction to a pyridoxylamino complex.

reversibly interact with the Schiff base linkage of protein bound PL to form a substituted aldamine in a manner analogous to that shown in Figure 7.30.

Vitamin B_6 also can be converted to biologically inactive compounds by reactions with free radicals. Hydroxyl radicals generated during degradation of AA can directly attack the C^6 position of PN to form the 6-hydroxy-derivative [138]. Presumably this reaction could occur with all other forms of vitamin B_6. 6-Hydroxypyridoxine totally lacks vitamin B_6 activity.

7.8.5.3 Bioavailability of Vitamin B_6

Many factors influence the bioavailability of vitamin B_6 [53]. The bioavailability of vitamin B_6 from a typical mixed diet has been estimated to be ~75% for adult humans [141]. Dietary PL, PN, PM, PLP, PMP, and PNP, if the latter is present, appear to be efficiently absorbed and effectively function in vitamin B_6 metabolism. Schiff base forms of PL, PLP, PM, and PMP dissociate in the acidic environment of the stomach and exhibit high bioavailability.

PN-glucoside and other glycosylated forms of vitamin B_6 are partially utilized in humans. The mean bioavailability of PN-glucoside is 50–60% relative to PN, although wide variation is observed among individuals. The importance of the incomplete bioavailability of PN-glucoside in human diets depends largely on the total quantity of vitamin B_6 consumed and the selection of foods. However, even foods with high percentages of PN-glucoside can be quite effective sources of dietary vitamin B_6

because of this partial bioavailability. PN-glucoside bioavailability varies markedly among animal species. It is essential that analytical methods for vitamin B_6 are able to detect the glycosylated forms and, ideally, provide a specific measurement of their relative quantities.

Determining the bioavailability of PL or PLP in the form of a pyridoxylamino compound (e.g., pyridoxyllysine, Figure 7.33) is not simple. In contrast to the Schiff base forms, pyridoxylamino forms have a very stable reduced linkage between PL or PLP and the lysine ε-amino group. Because of this stable covalent linkage, there is little or no dissociation of the complex under extraction conditions typically used in vitamin B_6 analysis. Thus, this stable covalent linkage of PL or PLP to protein appears as a mode of vitamin B_6 degradation and is seen as a loss of measurable vitamin B_6. However, as stated above, from the bioavailability standpoint, the of PLP pyridoxyllysyl residues in to dietary protein had a bioavailability of ~50% [47], suggesting that mammals can partially enzymatically cleave the pyridoxyl–lysyl bond and free the PLP moiety.

7.8.5.4 Measurement of Vitamin B_6

Vitamin B_6 can be measured by microbiological assay methods or by HPLC [51]. Microbiological assays for total vitamin B_6 can be performed using the yeasts *Saccharomyces uvarum* or *Kloeckera brevis*. Yeast growth assays involve a prior acid hydrolysis to extract vitamin B_6 from food, and to hydrolyze phosphate esters and β-glucosides. Care must be taken when using microbiological assays because the organisms used may underestimate PM. HPLC methods are based mainly on reverse phase or ion exchange separation with fluorometric detection, and direct measurement of each form of the vitamin can be achieved.

7.8.6 Folate

7.8.6.1 Structure and General Properties

The generic term "folate" refers to the class of pteridine derivatives having chemical structure and nutritional activity similar to that of folic acid (pteroyl-L-glutamic acid). The various components of this class are designated as "folates." The use of "folacin" and "folic acid" as generic terms is no longer recommended. Folic acid consists of L-glutamic acid that is coupled through its α-amino group to the carboxyl group of para-aminobenzoic acid that, in turn, is linked to a 2-amino-4-hydroxypteridine (Figure 7.34). In folic acid, the pteridine moiety exists in oxidized form. All folates contain an amide-like structure involving N^3 and C^4 that resonates between the two forms shown in Figure 7.35.

Folic acid (pteroyl-L-glutamic acid) exists naturally only in trace quantities. The major naturally occurring forms of folate in materials of plant, animal, and microbial sources are polyglutamyl species of 5,6,7,8-tetrahydrofolates (H_4folates) (Figure 7.34), in which two double bonds of the pteridine ring system are reduced. Small amounts of 7,8-dihydrofolates (H_2folates) also exist naturally (Figure 7.34). Metabolically, H_4folates are mediators of one-carbon transport and transformations, that is, the transfer, oxidation, and reduction of one-carbon units, which account for the presence of folates having various one-carbon substituted forms in living cells. One-carbon substituents can exist on either the N^5 or N^{10} positions (predominantly as methyl or formyl groups), or as methylene ($-CH_2-$) or methenyl ($-CH=$) units bridging between N^5 and N^{10} (Figure 7.34). Many of the naturally occurring folates in plant and animal tissues, and in foods derived from plant and animal sources, have a side chain of 5–7 glutamate residues with γ-peptide linkages. It is generally assumed that ~50–80% of naturally occurring dietary folate exists in polyglutamyl form, depending on the pattern of food selection. Beginning in1998, the federally mandated addition of folic acid to most cereal grain foods (e.g., flours, enriched bread, rolls, pasta, rice, etc.) changed this pattern so that 25–50% of folate intake often is obtained as synthetic folic acid (pteroyl-L-glutamate) in typical diets. However, limited data exist concerning the distribution of the various folates in individual foods or whole diets. All folates, regardless of oxidation state of the pteridine ring system, N^5 or

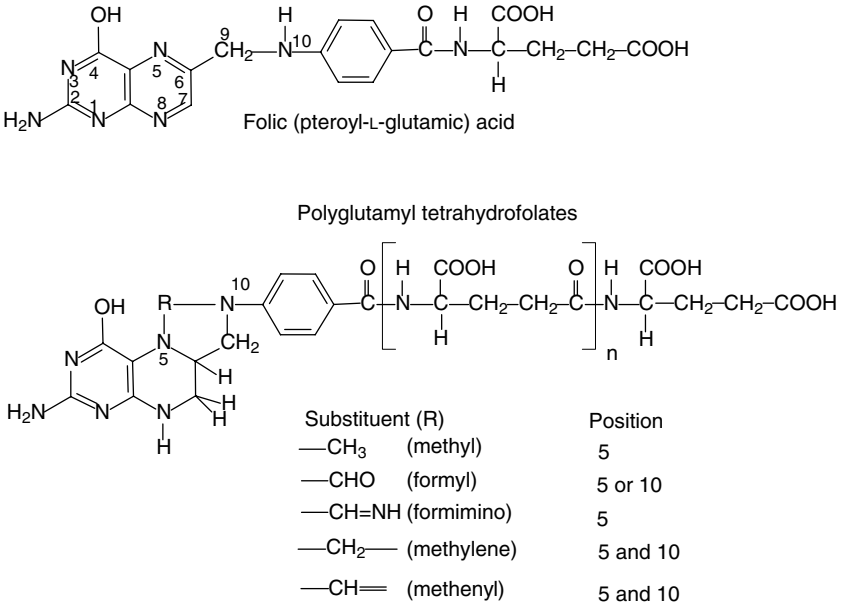


FIGURE 7.34 Structures of folates.

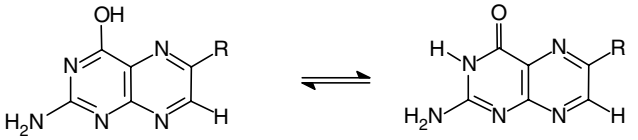


FIGURE 7.35 Resonance of the 3,4-amide site of folate pteridine ring system. The fully oxidized pteridine system of folic acid is shown; H_4folates and H_2folates exhibit identical behavior.

TABLE 7.25
pK_a Values for Ionizable Groups of Folates

Folate Compound	Amide[b]	N^1	N^5	N^{10}	α-COOH	γ-COOH
5,6,7,8-H_4folate	10.5	1.24	4.82	−1.25	3.5	4.8
7,8-H_2folate	9.54	1.38	3.84	0.28	ND[a]	ND[a]
Folic acid	8.38	2.35	<−1.5	0.20	ND[a]	ND[a]

[a] ND, not determined due to insufficient solubility. It is assumed that the pK_a values of these carboxyl groups are similar for all folates.
[b] Amide refers to dissociation of the N^3–C^4 amide-like site.

Source: Adapted from Poe, M. (1977). *J. Biol. Chem.* 252:3724–3728.

N^{10} one-carbon substituent, or polyglutamyl chain length, exhibit vitamin activity. Many structural analogs of folates, such as those with a 4-amino group, are potent antagonists used in chemotherapy for cancer and autoimmune disease.

Folates undergo changes in ionic form as a function of pH (Table 7.25) [112]. Changes in charge of the pteridine ring system account, in part, for the pH dependence of folate stability, UV absorption spectra, and the pH-dependent behavior of folates during chromatographic separation.

Asymmetric carbons (glutamyl α-carbon of all folates and pteridine C^6 of H_4folates) can each exist in either of two configurations. The glutamic acid moiety must be in the L isomeric form for vitamin activity, while C^6 must be in the *S* isomeric form for tetrahydrofolates to exhibit vitamin activity. Tetrahydrofolates synthesized by chemical reduction of folic acid contain a racemic carbon at C^6 while those reduced enzymatically are fully in the S configuration. Since the 6S configuration is required for vitamin activity in animals (including humans) and in microbiological assays for folate, racemic (6R + 6S) preparations of H_4folates only exhibit 50% nutritional activity.

The 5-formyl or 10-formyl folates have an aldehyde group as the one-carbon substituent. Formyl forms of H_4folate are interconvertible through the 5,10-methenyl intermediate. Formation of the methenyl species from either 5-formyl or 10-formyl-H_4folate is favored only at pH < 2; thus this form is but a minor constituent of folates in most foods. The transient existence of 5,10-methenyl-H_4folate at pH > 2 accounts for the conversion of 10-formyl-H_4folate to the more stable 5-formyl-H_4folate when heated in weak acid, and for the pH-dependent formation of 10-formyl-H_4folate from 5-formyl-H_4folate [119].

Large differences in stability exist among the various H_4folates as a result of the influence of the one-carbon substituent on susceptibility to oxidative degradation. In most cases, folic acid (with the fully oxidized pteridine ring system) exhibits substantially greater stability than the H_4folates or H_2folates. The order of stability of the H_4folates is 5-formyl-H_4folate $>$ 5-methyl-H_4folate $>$ 10-formyl-H_4folate $\geq$ H_4folate. Stability of each folate is dictated only by the chemical nature of the pteridine ring system, with no influence of polyglutamyl chain length. The inherent differences in stability among folates, as well as chemical and environmental variables influencing folate stability, will be discussed further in the next section.

All folates are subjected to oxidative degradation, although the mechanism and the nature of the products vary among the various chemical species of the vitamin. Reducing agents such as AA and thiols exert multiple protective effects on folates through their actions as oxygen scavengers, reducing agents, and free radical scavengers.

Aside from molecular oxygen, other oxidizing agents found in foods can have deleterious effects on folate stability. For example, at concentrations similar to those used for antimicrobial treatments, hypochlorite causes oxidative cleavage of folic acid, H_2folate, and H_4folate to nutritionally inactive products. Under the same oxidizing conditions, certain other folates (e.g., 5-methyl-H_4folate) are converted to forms that may retain at least partial nutritional activity. Light is also known to promote the cleavage of folates, although the mechanism has not been determined.

Before the initiation of fortification with folic acid in the United States, folate was frequently one of the most limiting of the vitamins in human diets. This remains the case in most other countries that do not practice the addition of folic acid to food. The frequent insufficiency of naturally occurring dietary folate is mainly due to: (1) poor diet selection, especially with respect to foods rich in folate (e.g., fruits, especially citrus, green leafy vegetables, and organ meats); (2) losses of folate during food processing and home preparation by oxidation, leaching, or both; and (3) incomplete bioavailability of many naturally occurring forms of folate in many human diets [54].

Folic acid, because of its excellent stability, is the sole form of folate added to foods, and it is also used in vitamin pills. In clinical situations requiring use of a reduced folate, 5-formyl-H_4folate is employed because of its stability (similar to folic acid), and 5-methyl-H_4folate also is available in a few nutritional supplements.

7.8.6.2 Stability and Modes of Degradation

7.8.6.2.1 Folate Stability

Folic acid exhibits excellent retention during the processing and storage of fortified foods and premixes [48]. As shown in Tables 7.2 and 7.3, little degradation of this form of the vitamin occurs during extended low moisture storage. Similar good retention of added folic acid has been observed during the retorting of fortified infant formulas and medical formulas.

TABLE 7.26
Effect of Cooking on Folate Content of Selected Vegetables

Vegetable (Boiled 10 min in Water)	Total Folate[a] (μg/100 g Fresh wt.)		
	Raw	Cooked	Folate in Cooking Water
Asparagus	175 ± 25	146 ± 16	39 ± 10
Broccoli	169 ± 24	65 ± 7	116 ± 35
Brussels sprouts	88 ± 15	16 ± 4	17 ± 4
Cabbage	30 ± 12	16 ± 8	17 ± 4
Cauliflower	56 ± 18	42 ± 7	47 ± 20
Spinach	143 ± 50	31 ± 10	92 ± 12

[a] Mean ± SD, $n = 4$.

Source: Adapted from Leichter, J., V. P. Switzer, and A. F. Landymore (1978). *Nutr. Rep. Int*. 18:475–479.

Many studies have shown the potential for extensive losses of folate during processing and home preparation of foods. In addition to susceptibility to oxidative degradation, folates are readily leached from foods by aqueous cooking media (Table 7.26) [88]. By either means, large losses of naturally occurring folate can occur during food processing and preparation. The overall loss of folate from a food depends on the extent of leaching, forms of folate present, and the nature of the chemical environment (catalysts, oxidants, pH, buffer ions, etc.). Thus, folate retention is difficult to predict for a given food.

7.8.6.2.2 Degradation Mechanisms

The mechanism of folate degradation depends on the form of the vitamin and the chemical environment. As mentioned previously, folate degradation generally involves changes at the $C^9—N^{10}$ bond, the pteridine ring system, or both. Folic acid, H_4folate, and H_2folate can undergo $C^9—N^{10}$ cleavage, and resulting inactivation in the presence of either oxidants or reductants [97]. Dissolved SO_2 has been found to cause cleavage of certain folates, but few other reducing agents relevant to foods can induce such cleavage. There is only slight direct oxidative conversion of H_4folate to H_2folate or folic acid.

It is well known that oxidative cleavage (Figure 7.36) of H_4folates, H_2folate, and to a lesser extent, folic acid, yields nutritionally inactive products (*p*-aminobenzoylglutamate and a pterin). The mechanism of oxidation and the nature of the pterin produced during oxidative cleavage of H_4folate vary with pH.

The major naturally occurring form of folate in many foods, 5-methyl-H_4folate, can degrade by conversion to at least two products (Figure 7.37) [115]. The first has been identified tentatively as 5-methyl-5,6-dihydrofolate (5-methyl-H_2folate), which retains vitamin activity since it can be readily reduced back to 5-methyl-H_4folate by weak reductants such as thiols or ascorbate. 5-Methyl-H_2folate undergoes cleavage of the $C^9—N^{10}$ bond in acidic medium, which causes loss of vitamin activity. Some data suggest that a rearrangement of the pteridine can occur to form a pyrazino-*s*-triazine (Figure 7.37; 73,115). An alternate product of 5-methyl-H_4folate degradation appears to be 4α-hydroxy-5-methyl-H_4folate, which actually may be the predominant degradation product in some foods and other biological systems. Many aspects of chemical mechanisms of processes involved in 5-methyl-H_4folate degradation remain to be determined.

Blair et al. [9] reported that the oxidation of 5-methyl-H_4folate oxidation is strongly pH dependent. Stability (as monitored by oxygen uptake) increases as pH is reduced from 6 to 4, this range

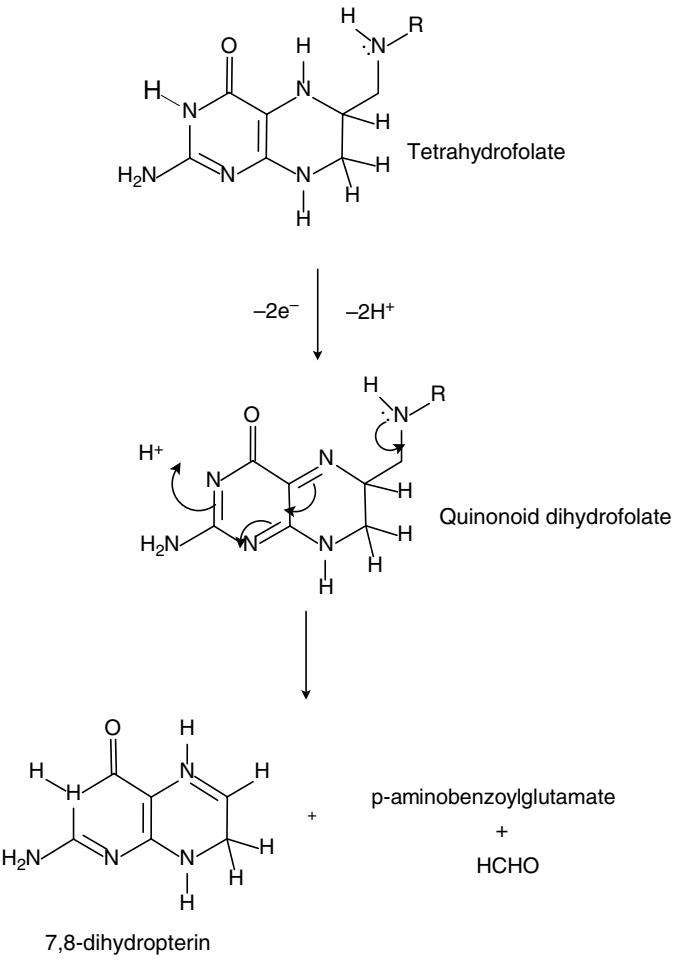


FIGURE 7.36 Proposed mechanisms for oxidative degradation of H_4folate. (Adapted from Reed, L. S. and M. C. Archer (1980). *J. Agric. Food Chem*. 28:801–805.)

corresponding to the range of protonation of the N^5 position. Contrary results have been reported [100], and factors responsible for this contradiction have not been determined.

In certain foods, including various animal and plant tissues, 10-formyl-H_4folate may account for as much as one-third of the total folate. Oxidative degradation of 10-formyl-H_4folate can occur either by oxidation of the pteridine moiety to yield 10-formyl-H_2folate or 10-formyl-folate or by oxidative cleavage to form a pterin and *N*-formyl-*p*-aminobenzoylglutamate (Figure 7.38). Both 10-formyl-H_2folate or 10-formyl-folate retain nutritional activity while the cleavage products do not. The detection of 10-formyl-H_2folate or 10-formyl-folate in a variety of foods [84,110] suggests that oxidation of 10-formyl-H_4folate occurs readily during food storage preparation and processing. Factors that influence the relative importance of these oxidative pathways in foods have not been determined. In contrast to 10-formyl-H_4folate, 5-formyl-H_4folate exhibits excellent thermal and oxidative stability.

7.8.6.2.3 Factors Affecting Folate Stability

Many studies have been conducted to compare the relative stability of folates in buffered solution as a function of pH, oxygen concentration, and temperature. Stability of folates in complex foods is less well understood.

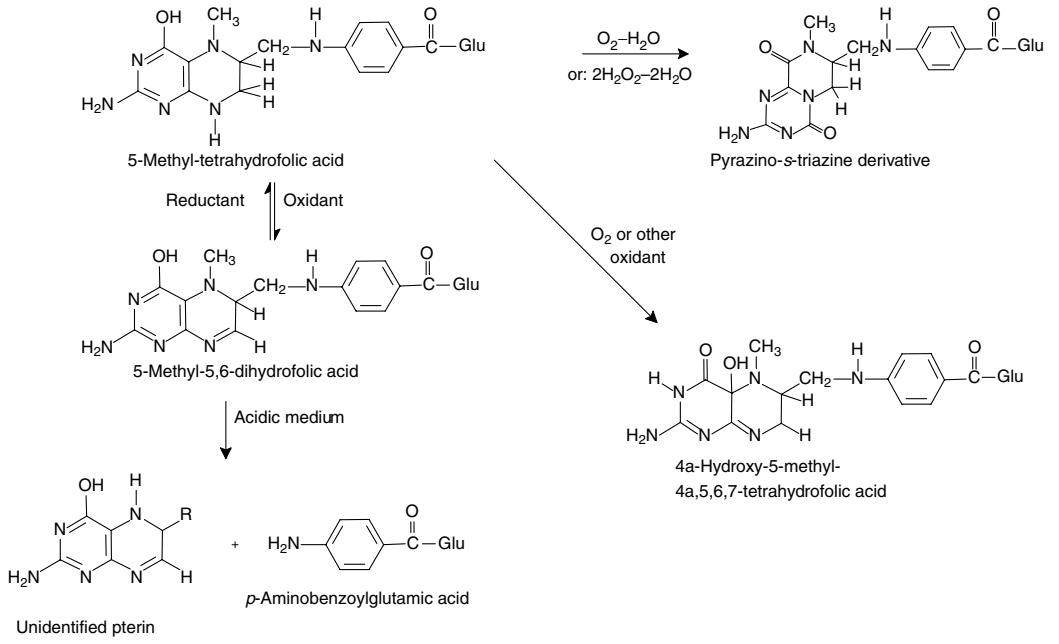


FIGURE 7.37 Proposed mechanisms for oxidative degradation 5-methyl-H_4folate.

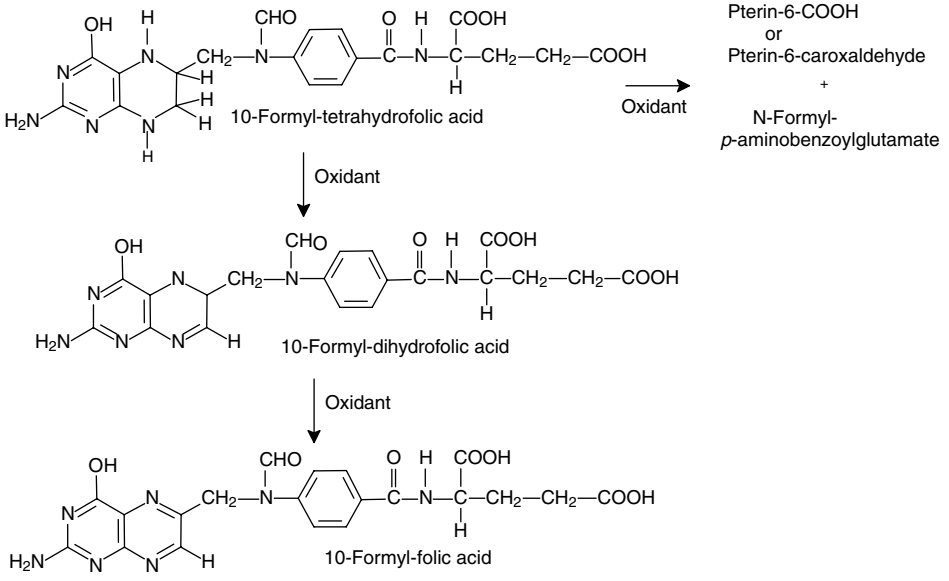


FIGURE 7.38 Proposed mechanisms for oxidative degradation of 10-formyl-H_4folate.

Maximal stability of H_4folate is observed between pH 8–12 and 1–2, while the stability is minimal between pH 4 and 6. However, even in the favorable pH zones, H_4folate is extremely unstable. H_4folates having a substituent at the N^5 position exhibit much greater stability than does unsubstituted H_4folate. This suggests that the stabilizing effect of the N^5 methyl group is due, at least in part, due to steric hindrance in restricting access of oxygen or other oxidants to the pteridine ring. The stabilizing effect of the N^5 substituent is more pronounced with 5-formyl-H_4folate than with

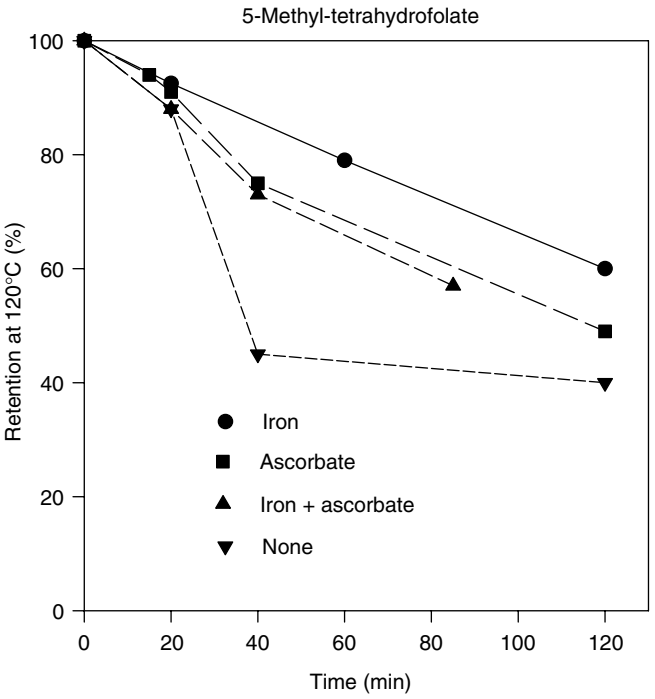


FIGURE 7.39 Thermal processing effects on 5-methyl-H_4folate in retorted cans of liquid model food systems simulating infant formula. The model system consisted of 1.5% (w/v) potassium caseinate and 7% (w/v) lactose in 0.1 M phosphate buffer, pH 7.0. When present, iron was added at 6.65 mg/100 ml ferrous sulfate heptahydrate and ascorbate was added as 6.38 mg/100 ml sodium ascorbate. Initial concentration of folates was 10 μg/ml. (From Day, B. P. F. and J. F. Gregory (1983). *J. Food Sci.* 48:581–587, 599.)

5-methyl-H_4folate, and both exhibit much greater stability than H_4folate or 10-formyl-H_4folate. Under conditions of low oxygen concentration, 5-methyl-H_4folate and folic acid exhibit similar stability during thermal processing.

The rate of oxidation of 5-methyl-H_4folate is dependent on the concentration of dissolved oxygen in accord with a second-order or pseudo-first-order relationship. In relatively anaerobic conditions, the presence of added components such as ascorbate, ferrous iron, and reducing sugar all tend to improve the oxidative stability of folic acid and 5-methyl-H_4folate. These components apparently function by reducing the concentration of dissolved oxygen through their own oxidation reactions (Figure 7.39). Thus, complex foods may contain components that influence folate stability by consuming oxygen, acting as reducing agents, or both.

Barrett and Lund [6] reported that the rate constants for thermal degradation of 5-methyl-H_4folate in neutral buffer solution under both aerobic and anaerobic conditions were similar in magnitude (Table 7.27). The extent to which other folates conform to this behavior has not been determined. Clearly the loss of 5-methyl-H_4folate during food processing is minimized but not eliminated by minimizing oxygen availability [149].

The ionic composition of the medium also significantly influences the stability of most folates. Phosphate buffers accelerate oxidative degradation of folates, while this effect can be overcome by the addition of citrate ions. The frequent presence of Cu(II) as a contaminant in phosphate buffer salts, may explain this effect because metal catalysts are known to accelerate folate oxidations. For example, in aerobic solutions of 5-methyl-H_4folate in water, addition of 0.1 mM Cu(II) causes nearly a 20-fold acceleration in oxidation rate, although Fe(III) causes only a twofold increase [9]. Under anaerobic conditions, Fe(III) catalyzes oxidation of H_4pteridines (e.g., H_4folate) $\rightarrow$ H_2pteridines

TABLE 7.27
Reaction Rate Constants for the Degradation of 5-methyl-H_4folate by Oxidative and Nonoxidative Processes in 0.1 M Phosphate Buffer, pH 7.0[a,b]

Temp (°C)	$k_{(O2+N2)}$ (Combined Oxidative + Nonoxidative, min^{-1})	k_{N2} (Nonoxidative, min^{-1})	k_{O2} (Oxidative, min^{-1})
40	0.004 ± 0.0002	0.0005 ± 0.00001	0.004 ± 0.00005
60	0.020 ± 0.0005	0.009 ± 0.0004	0.011 ± 0.0001
80	0.081 ± 0.010	0.046 ± 0.003	0.035 ± 0.009
92	0.249 ± 0.050	0.094 ± 0.009	0.155 ± 0.044

[a] Values are means ± 95% CI.

[b] Values for apparent first-order rate constants: k_{N2}, degradation by nonoxidative process (in N_2-saturated environment); k_{O2}, degradation by oxidative process (in O_2-saturated environment); $k_{(O2+N2)}$, degradation by both oxidative and nonoxidative processes.

Source: Barrett, D. M. and D. B. Lund (1989). *J. Food Sci.* 54:146–149.

(e.g., H_2folate) → fully oxidized pteridines (e.g., folic acid). Folates undergo degradation by superoxide ions [131,139], although the extent of such radical-mediated losses of folates in foods has not been determined.

Several reactive components of foods may accelerate degradation of folates. Dissolved SO_2 can cause reductive cleavage of folates, as stated previously. Exposure to nitrite ions contributes to the oxidative cleavage of 5-methyl-H_4folate and H_4folate. In contrast, nitrite reacts with folic acid to yield 10-nitroso-folic acid, a weak carcinogen. However, it is reassuring to note that foods containing nitrite do not generally contain folic acid and have low concentrations of other folates. The significance of the latter reaction in foods is minimal because folic acid does not occur significantly in foods containing nitrite. Oxidative degradation of folates by exposure to hypochlorite may yield significant losses of folates in certain foods.

7.8.6.3 Bioavailability of Folate in Foods

The absorption of folates takes place mainly in the jejunum and requires hydrolysis of the polyglutamyl chain by a specific peptidase (pteroylpolyglutamate hydrolase), followed by absorption via a carrier-mediated transport process [48,54]. Bioavailability of naturally occurring folate in foods is incomplete, often averaging 50% or less [125]. Moreover, the bioavailability of *naturally occurring* folates in most foods has not been fully determined under conditions of actual consumption, including the consequences of interactions among various foods. The mean bioavailability of polyglutamyl folates is typically 70% *relative to the monoglutamyl species*, which indicates the potentially rate-limiting nature of intestinal deconjugation. Although it has been reported based on early studies that the bioavailability of folic acid added to cereal grain products is only 30–60% [20], later investigations showed that folic acid is highly bioavailable in fortified cereal grain food products [111].

Factors responsible for incomplete bioavailability include: (1) effects of the food matrix, presumably through noncovalent binding of folates or entrapment in cellular structure; (2) possible degradation of labile H_4 folates in the acidic gastric environment; and (3) incomplete intestinal enzymatic conversion of polyglutamyl folates to the absorbable monoglutamyl forms. Many foods contain compounds that inhibit intestinal pteroylpolyglutamate hydrolase when studied *in vitro*; however, the significance of these effects with respect to *in vivo* folate bioavailability is unclear. Many raw fruits, vegetables, and meats also contain active conjugases capable of deconjugating polyglutamyl folates. Homogenization, freezing and thawing, and other procedures that disrupt cells

may release these enzymes and promote the deconjugation process. The extent to which this would improve bioavailability of dietary folates has not been determined. Little or no deconjugation of polyglutamyl folates occurs during food preparation and processing, unless endogenous conjugases are present.

7.8.6.4 Analytical Methods

Techniques potentially suitable for the measurement of folate in foods include microbiological growth methods, HPLC methods, and competitive-binding radioassay procedures [48]. Measurement of folate is complicated by the need to account for all forms of the vitamin, which could easily include several dozen compounds if each form of the folate nucleus existed in all possible combinations with several different polyglutamate chain lengths. Either ascorbate, a thiol reagent such as mercaptoethanol, or a combination of ascorbate and thiol are needed to stabilize folates during extraction and analysis.

Extraction of folate from food samples involves: (1) disruption of the food matrix and cellular structure by homogenization in a buffer solution; (2) heat (typically 100°C) to release folate from folate-binding proteins, to inactivate enzymes capable of catalyzing interconversion of folates, and to deproteinate the sample; (3) centrifugation to yield a clarified extract; and (4) treatment with a pteroylpolyglutamate hydrolase (conjugase), if the assay responds only to monoglutamyl or other short-chain folates. Other enzymes such as a protease or amylase may be useful in improving extraction of folate from certain foods. A need exists to standardize extraction and enzymatic pretreatment methods so interlaboratory precision and accuracy of folate assays can be improved.

Microbiological growth assays serve as the traditional method of folate analysis and are based on the nutritional requirements of microorganisms (*Lactobacillus casei*, *Pediococcus cerevisiae*, and *Streptococcus faecium*). *P. cerevisiae* and *S. faecium* (used in an AOAC official method) have little use in food analysis because they do not respond to all forms of the vitamin. In contrast, *L. casei* responds to all forms of folate and serves as the most appropriate test organism for microbiological assays of total folate in food. With appropriate control of pH in the growth medium, *L. casei* yields equivalent response to all forms of folate. Since foods typically contain several folates, verification of equivalent response in microbiological assays is essential.

Competitive-binding assays involve competition between folate in the sample or standard with radiolabeled folate for the binding site of a folate-binding protein, typically from milk. In spite of the speed and convenience of these assays, their application to food analysis is limited because of varying affinity for different forms of folate. Comparisons of competitive-binding assays with the *L. casei* method have yielded poor agreement, presumably for this reason.

Several HPLC methods have been developed for measurement of folates in foods and other biological materials. Several approaches to HPLC analysis are potentially suitable, although extractions, enzymatic pretreatments, and sample cleanup must be optimized to permit application to various types of foods. An important advance in HPLC is the use of affinity chromatography with a folate-binding protein column to yield a highly purified extract that is amenable to HPLC analysis [129]. HPLC analysis can be performed on extracted folates in intact polyglutamyl form or following enzymatic deconjugation to the monoglutamyl form [48,129]. Such HPLC methods sometimes yield results that are lower than those of *L. casei* assays [84], although this is not always the case [110]. It is not clear whether HPLC methods sometimes give an incomplete measurement or if the microbiological assay is subject to nonspecific responses. Liquid chromatography-mass spectrometry methods for the determination of food folate may improve analytical specificity.

The folate content of foods is highly variable, presumably as a function of climate and agricultural practices as well as postharvest handling, and susceptibility to losses by leaching and oxidation. Thus, mean values for folate content in food composition databases must be considered only as rough approximations. Furthermore, because of analytical limitations, especially with respect to extraction methods, much of the data used in such databases are of questionable accuracy.

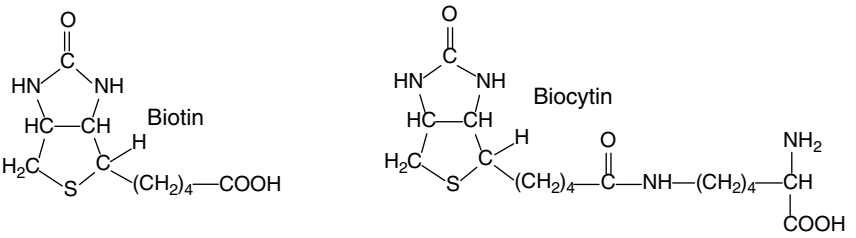


FIGURE 7.40 Structures of biotin and biocytin.

7.8.7 Biotin

7.8.7.1 Structure and General Properties

Biotin is a bicyclic, water-soluble vitamin that functions coenzymatically in carboxylation and transcarboxylation reactions. The two naturally occurring forms are free D-biotin and biocytin (*ε-N*-biotinyl-L-lysine) (Figure 7.40) [26]. Biocytin functions as the coenzyme form and actually consists of a biotinylated lysyl residue, formed by posttranslational biotinylation, that is, covalently incorporated in a protein chain of various carboxylases including a role in fatty acid synthesis. The ring system of biotin can exist in eight possible stereoisomers, only one of which (D-biotin) is the natural, biologically active form. Both free biotin and protein-bound biocytin exhibit biotin activity when consumed in the diet, whereas the naturally occurring catabolic products of biotin in animal tissues (*bis*-norbiotin and biotin sulfoxide) do not exhibit vitamin activity. Biotin is widely distributed in plant and animal products, and biotin deficiency is rare in normal humans.

7.8.7.2 Stability of Biotin

Biotin is very stable to heat, light, and oxygen. Extremes of high or low pH can cause degradation, possibly because they promote hydrolysis of the $-NH-C=O$ (amide) bond of the biotin ring system. Oxidizing conditions such as exposure to hydrogen peroxide can oxidize the sulfur to form biologically inactive biotin sulfoxide or sulfone. Losses of biotin during food processing and storage have been documented and summarized [69,92,160]. Such losses may occur by chemical degradation processes as mentioned above and by leaching of free biotin. Little degradation of biotin occurs during low moisture storage of fortified cereal products. Overall, biotin is quite well retained in foods.

The stability of biotin during storage of human milk also has been examined [101]. The biotin concentration of the milk samples did not change over 1 week at ambient temperature, 1 month at 5°C, or at −20°C or lower for 1.5 years.

7.8.7.3 Bioavailability

Relatively little is known about the bioavailability of biotin in foods. There appears to be sufficient biotin in normal diets that incomplete bioavailability usually has little adverse nutritional impact. Bacterial synthesis of biotin in the lower intestine provides an additional source of partially available biotin for humans. The majority of biotin in many foods exists as protein bound biocytin. This is released by biotinidase in pancreatic juice and in the intestinal mucosa to convert the bound biotin to the functionally active free form; however, some absorption of biotinyl peptides also may occur.

Absorption of biotin is almost totally prevented by the consumption of raw egg albumen which contains the biotin-binding protein avidin. Avidin is a tetrameric glycoprotein in egg albumen that is capable of binding one biotin per subunit. This protein binds biotin very tightly (dissociation constant $\sim 10^{-15}$ M) and resists digestion. Little or no avidin-bound biotin is absorbed. Chronic consumption of raw eggs or raw egg albumen will, thus, impair biotin absorption and can lead to deficiency. Small amounts of avidin in the diet have no nutritional consequence. The use of dietary avidin (or egg

TABLE 7.28
Bioavailabililty of Biotin in Feedstuffs for Pigs and Turkeys

	Biotin Bioavailability (%)	
Material	**Pigs (Ref. 126)**	**Turkeys (Ref. 99)**
Soybean meal	55.4	76.8
Meat and bone meal	2.7	N.D.[a]
Canola meal	3.9	65.4
Barley	4.8	19.2
Corn	4.0	95.2
Wheat	21.6	17.0
Supplemental biotin	93.5	ND
Sorghum	ND	29.5

[a] N.D., not determined.

albumen) permits the experimental development of biotin deficiency in laboratory animals. Cooking denatures avidin and eliminates its biotin-binding properties.

While little information exists regarding the bioavailability of biotin in humans, much more is known about its bioavailability in animal feedstuffs. As shown in Table 7.28 (99,126), the bioavailability of biotin is low in some materials.

7.8.7.4 Analytical Methods

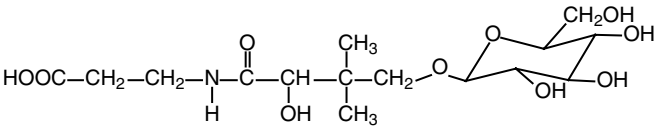
Measurement of biotin in foods is performed by microbiological assay (usually with *Lactobacillus plantarum*) or by various ligand-binding procedures involving avidin as the binding protein. Several HPLC methods also have been developed. Most of these involve the use of an avidin-binding procedure to provide sensitivity and increased sensitivity. The microbiological HPLC and ligand-binding assays respond to free biotin and biocytin, but biocytin cannot be determined unless it is first released from the protein by cleavage of the peptide bond by enzymatic or acid hydrolysis [101,160]. Care should be taken because acid hydrolysis can degrade a substantial proportion of the biotin [101]. The existence of nutritionally inactive biotin analogs, such as *bis*-norbiotin and biotin sulfoxide detected in some animal tissues and in human urine, may complicate analyses. Such analogs may respond in avidin-binding procedures and certain microbiological assays. Separation of the biotin derivatives by HPLC prior to the avidin-binding assay of alleviates such problems by allowing their individual measurement.

7.8.8 Pantothenic Acid

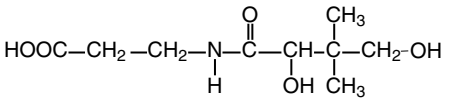
7.8.8.1 Structure and General Properties

Pantothenic acid, or D-*N*-(2,4-dihydroxy-3,3-dimethyl-butyryl-β-alanine), is a water soluble vitamin composed of β-alanine in amide linkage to 2,4-dihydroxy-3,3-dimethyl-butyric (pantoic) acid (Figure 7.41). Pantothenic acid functions metabolically as a component of coenzyme A and as a covalently bound prosthetic group (without the adenosyl moiety of coenzyme A) of acyl carrier protein in fatty acid synthesis. Formation of a thioester derivative of coenzyme A with organic acids facilitates a wide variety of metabolic processes that mainly involve addition or removal of acyl groups, in an array of biosynthetic and catabolic reactions. Pantothenic acid is essential for all living things and is distributed widely among meats, cereal grains, eggs, milk, and many fresh vegetables.

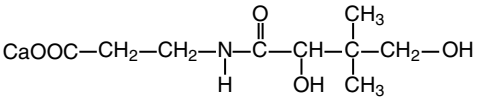
Pantothenic acid in many foods and most biological materials is mainly in the form of coenzyme A, the majority of which exists as thioester derivatives of a wide variety of organic acids. Although analytical data are quite limited with respect to the free and coenzyme A forms of pantothenic acid in



Pantothenyl-β-D-glucoside



Pantothenic acid



Calcium pantothenate

FIGURE 7.41 Structure of various forms of pantothenic acid.

foods, free pantothenic acid has been found to account for only half of the total content of this vitamin in beef muscle and peas [59]. Coenzyme A is fully available as a source of pantothenic acid because it is converted to free pantothenic acid in the small intestine by the action of alkaline phosphatase and an amidase. Intestinal absorption occurs through a carrier-mediated absorption process.

Synthetic pantothenic acid is used in food fortification and in vitamin supplements in the form of calcium pantothenate. This compound is a white crystalline material that exhibits greater stability and is less hygroscopic than the free acid. Panthenol, the corresponding alcohol, also has been used as a feed supplement for animals. Panthenol also is used as an ingredient in certain shampoos for apparent physical (i.e., lubricating) effects, rather than nutritional effects, when applied to hair.

7.8.8.2 Stability and Modes of Degradation

In solution, pantothenic acid is most stable at pH 5–7. Pantothenic acid exhibits relatively good stability during food storage, especially at reduced water activity. Losses occur in cooking and thermal processing in proportion to the severity of the treatment and extent of leaching, and these range from 30 to 80%. Although the mechanism of thermal loss of pantothenic acid has not been fully determined, an acid- or base-catalyzed hydrolysis of the linkage between β-alanine and the 1,4-dihydroxy,3,3-butyryl-carboxylic acid group appears likely. The pantothenic acid molecule is otherwise quite unreactive and interacts little with other food components. Coenzyme A is susceptible to the formation of mixed disulfides with other thiols in foods; however, this exerts little effect on the net quantity of available pantothenic acid.

Degradation of pantothenic acid during thermal processing conforms to first-order kinetics [59]. Rate constants for degradation of free pantothenic acid in buffered solutions increase with decreasing pH over the range of pH 6.0–4.0, while the energy of activation decreases over this range. The rates of degradation reported for pantothenic acid are substantially less than those for other labile nutrients (e.g., thiamin). These findings suggest that losses of pantothenic acid reported in other studies of food processing may be predominantly due to leaching rather than actual destruction. The net result would be the same, however.

7.8.8.3 Bioavailability

The mean bioavailability of pantothenate in a mixed diet has been reported to be ~50% [141]. There is little concern regarding any adverse consequences of this incomplete bioavailability because

pantothenic acid intake is generally adequate. No evidence of nutritionally significant problems of incomplete bioavailability has been reported, and the complexed coenzymic forms of the vitamin are readily digested and absorbed.

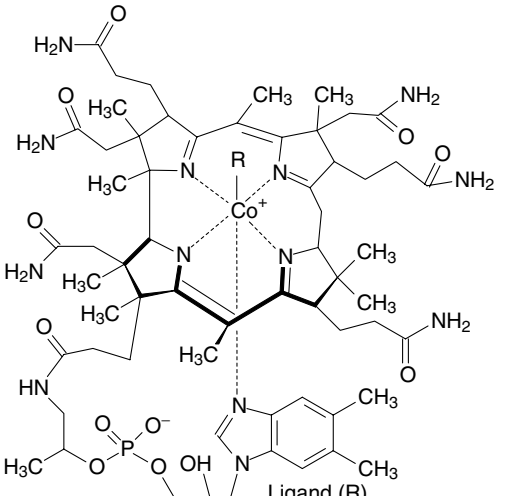
7.8.8.4 Analytical Methods

Pantothenic acid in foods may be measured primarily by microbiological assay using *L. plantarum* or by radioimmunoassay [156]. A key factor that affects the validity of pantothenic acid analysis is the pretreatment needed to release bound forms of the vitamin. Various combinations of proteases and phosphatases have been used to release pantothenic acid from the many coenzyme A derivatives and protein bound forms.

7.8.9 Vitamin B_{12}

7.8.9.1 Structure and General Properties

Vitamin B_{12} is the generic term for the group of compounds (cobalamins) having vitamin activity similar to that of cyanocobalamin. These compounds are corrinoids, tetrapyrrole structures in which a cobalt ion (Co) is coordinately covalently bonded to the four pyrrole nitrogens. The fifth coordinate covalent bond to Co is with a nitrogen of the dimethylbenzimidazole moiety, while the sixth position may be occupied by cyanide, a 5′-deoxyadenosyl group, a methyl group, glutathione, water, a hydroxyl ion, or other ligands such as nitrite, ammonia or sulfite (Figure 7.42). All forms of vitamin B_{12} shown in Figure 7.42 exhibit vitamin B_{12} activity. Cyanocobalamin, a synthetic form of vitamin B_{12} used in food fortification and nutrient supplements, exhibits superior stability and is readily available commercially. The coenzyme forms of vitamin B_{12} are methylcobalamin and 5′-deoxyadenosylcobalamin. Methylcobalamin functions coenzymatically in the transfer of a methyl



Ligand (R)	B_{12} Form
–CN	Cyanocobalamin
–OH	Hydroxocobalamin
$-H_2O$	Aquocobalamin
–glutathione	Glutathionylcobalamin
$-CH_3$	Methylcobalamin
–5'-Deoxyadenosine	5'-Deoxyadenosyl-cobalamin

FIGURE 7.42 Structure of various forms of vitamin B_{12}.

group (from 5-methyl-tetrahydrofolate) in methionine synthase, while 5′-deoxyadenosylcobalamin serves as the coenzyme in an enzymatic rearrangement reaction catalyzed by methylmalonyl-CoA mutase [136]. Little or no naturally occurring cyanocobalamin exists in foods; in fact, the original identification of vitamin B_{12} as cyanocobalamin involved its formation as an artifact of the isolation procedure. Cyanocobalamin has a reddish color in the crystalline state and in solution. This coloration may pose a limitation in the possible addition of cyanocobalamin to certain foods, especially lightly colored products (e.g., white bread).

Unlike other vitamins that are synthesized primarily in plants, only microorganisms produce cobalamins. Certain legumes have been reported to absorb small amounts of vitamin B_{12} produced by bacteria associated with root nodules, but little enters the seeds [103]. Most plant-derived foods are devoid of vitamin B_{12} unless contaminated by fecal material, for example, from fertilizer [65,66]. The vitamin B_{12} in most animal tissues consists mainly of the coenzyme forms, methylcobalamin and 5′-deoxyadenosylcobalamin, in addition to aquocobalamin.

Approximately 20 naturally occurring analogs of vitamin B_{12} have been identified. Some of these have no biological activity in mammals, some may be vitamin B_{12} antagonists and others exhibit at least partial vitamin activity but are often poorly absorbed.

7.8.9.2 Stability and Modes of Degradation

Under most conditions of food processing, preservation, and storage, there is little nutritionally significant loss of vitamin B_{12}. Cyanocobalamin added to breakfast cereal products has been reported to undergo an average loss of 17% during processing, with an additional 17% loss during storage for 12 months at ambient temperature [137]. In studies of the processing of fluid milk, 96% mean retention has been observed during high HTST pasteurization and similar retention (>90%) was found in milk processed using various modes of ultrahigh temperature (UHT) processing [43]. Although refrigerated storage of milk has little impact on vitamin B_{12} retention, storage of UHT-processed milk at ambient temperature for up to 90 days causes progressive losses that can approach 50% of the initial vitamin B_{12} concentration [17]. Sterilization of milk for 13 min at 120°C has been reported to cause only 23% retention of vitamin B_{12} [76], but prior concentration (as in production of evaporated milk) contributes to more severe losses. This indicates the potential for substantial losses of vitamin B_{12} during prolonged heating of foods at or near neutral pH. Typical oven heating of commercially prepared convenience dinners has been shown to yield 79–100% retention of vitamin B_{12}.

Ascorbic acid has long been known to accelerate the degradation of vitamin B_{12}, although this may be of little practical significance because foods containing vitamin B_{12} usually do not contain significant amounts of AA. Douglass et al. [35] examined the influence of the use of ascorbate or erythorbate in curing solutions for ham and found that these compounds have no influence on vitamin B_{12} retention. Thiamin and nicotinamide in solution can accelerate degradation of vitamin B_{12}, but the relevance of this phenomenon to foods is unclear.

The mechanism of vitamin B_{12} degradation has not been fully determined, in part because of the complexity of the molecule and the very low concentration in foods. Photochemical degradation of vitamin B_{12} coenzymes yields aquocobalamin. Such a reaction interferes with experimental studies of B_{12} metabolism and function, but this conversion has no influence on the total vitamin B_{12} activity of foods because aquocobalamin retains vitamin B_{12} activity. The overall stability of vitamin B_{12} is greatest at pH 4–7. Exposure to acid causes the hydrolytic removal of the nucleotide moiety, and additional fragmentation occurs as the severity of the acidic conditions increases. Exposure to acid or alkaline conditions causes hydrolysis of amides, yielding biologically inactive carboxylic acid derivatives of vitamin B_{12}.

Interconversions among cobalamins can occur through exchange of the ligand bonded to the Co atom. For example, bisulfite ion causes conversion of aquocobalamin to sulfitocobalamin, while similar reactions can occur to form cobalamins substituted with ammonia, nitrite, or hydroxyl ions. These reactions have little influence on the net vitamin B_{12} activity of foods.

7.8.9.3 Bioavailability

The bioavailability of vitamin B_{12} has been examined mainly in the context of the diagnosis of vitamin B_{12} deficiency associated with malabsorption. Little is known about the influence of food composition on the bioavailability of vitamin B_{12}. Several studies have shown that pectin and, presumably, similar gums reduce vitamin B_{12} bioavailability in rats. The significance of this effect in humans remains unclear. Although little or no vitamin B_{12} is present in most plants, certain forms of algae do contain significant quantities of the vitamin. Forms of algae are not recommended as a source of vitamin B_{12} because of its very low bioavailability [25].

In normal human beings, absorption of vitamin B_{12} from eggs has been shown to be less than half that of cyanocobalamin administered in the absence of food [33]. Similar results have been obtained regarding vitamin B_{12} bioavailability in studies with fish and various meats [32,34]. Certain individuals are marginally deficient in vitamin B_{12} because of poor protein digestion and incomplete release of cobalamins from the food matrix even though they absorb the pure compound normally [18]. Such malabsorption of vitamin B_{12} from food is most prevalent in the elderly. Recent studies show that cyanocobalamin added to bread or milk is well absorbed by elderly people, which suggests that fortification of these products is technically feasible [122].

7.8.9.4 Analytical Methods

The concentration of vitamin B_{12} in foods is determined primarily by microbiological growth assays using *Lactobacillus leichmannii* or by radioligand-binding procedures [38]. Although the various forms of vitamin B_{12} can be separated chromatographically, HPLC methods are not readily suitable for food analysis because of the very low concentrations typically found. Early types of radioligand-binding assays for vitamin B_{12} in clinical specimens and foods were often inaccurate because the binding protein employed could bind to active forms of vitamin B_{12} as well as biologically inactive analog. The specificity of such assays has been greatly improved through the use of a vitamin B_{12}-binding protein (termed "intrinsic factor," generally from pig stomach) that is specific for the biologically active forms of the vitamin. Microbiological assays with *L. leichmannii* may be subject to interference if samples contain high concentrations of deoxyribonucleosides.

Food samples are generally prepared by homogenization in a buffered solution, followed by incubation at elevated temperature ($\sim$60°C) in the presence of papain (a protease) and sodium cyanide. This treatment releases protein bound forms of vitamin B_{12} and converts all cobalamins to the more stable cyanocobalamin form. Conversion to cyanocobalamin also improves the performance of assays that may differ in response to the various forms of the vitamin.

7.9 CONDITIONALLY ESSENTIAL VITAMIN-LIKE COMPOUNDS

7.9.1 Choline and Betaine

Choline (Figure 7.43) exists in all living things as the free compound and as a constituent of a number of cellular components including phosphatidylcholine (the most prevalent dietary source of choline), sphingomyelin, and acetylcholine. Although choline synthesis occurs in humans and other mammals, there is a growing body of evidence that an adequate supply of dietary choline is also required [45]. Thus, a nutritional requirement recently was defined for choline [70], However, healthy individuals consuming a varied diet rarely have inadequate choline intakes because choline exists in abundance (as choline, phosphocholine, and the membrane constituents sphingomyeline and phosphatidylcholine) in many food sources. Choline is used as chloride and bitartrate salts in fortification of infant formulas. It is not ordinarily added to other foods except as an ingredient, for

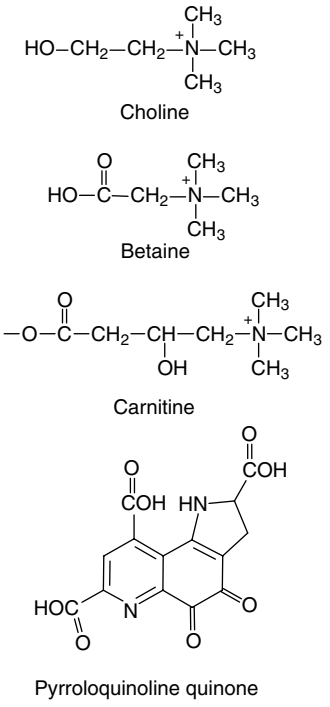


FIGURE 7.43 Structure of choline, betaine, carnitine, and pyrroloquinoline quinone.

example, phosphatidylcholine as an emulsifier. Choline is a very stable compound. No significant loss of choline during food storage, handling, processing, or preparation.

Betaine (*N*-trimethylglycine, Figure 7.43) is a component in the metabolic breakdown of choline. It occurs naturally in the diet and is especially high in beets, wheat, spinach, shrimp, and related food sources [159]. Betaine serves metabolically as an alternative to 5-methyl-H_4folate in a reaction that converts homocysteine to methionine for protein synthesis and, after formation of *S*-adenosylmethionine (SAM), many cellular methylation reactions. This process helps conserve methionine, control homocysteine levels, and facilitate SAM-dependent methylation processes in a manner that does not depend on a steady supply of folate. Because betaine is obtained from common foods and is generated *in vivo* from the generally ubiquitous choline, there is rarely a metabolic limitation in betaine. In situations in which plasma homocysteine is elevated for nutritional or genetic reasons, supplemental betaine is occasionally administered, along with vitamin supplements (B_6, B_{12}, and folic acid), in an effort to maximize the conversion of homocysteine to methionine.

7.9.2 Carnitine

Carnitine (Figure 7.43) can be synthesized by the human body; however, certain individuals appear to benefit from additional dietary carnitine [45]. No nutritional requirements have been established for carnitine. Although little or no carnitine is found in plants and plant products, it is widely distributed in foods of animal origin. Carnitine functions metabolically in the transport of organic acids across biological membranes and, thus, facilitates their metabolic utilization and disposal. Carnitine also facilitates transport of certain organic acids to lessen the potential for toxicity in certain cells. In animal-derived foods, carnitine exists in free and acylated form. The acyl carnitines occur with various organic acids esterified to the C-3 hydroxyl group. Carnitine is highly stable and undergoes little or no degradation in foods.

Synthetic carnitine is used in certain clinical applications as the biologically active L-isomer. D-Carnitine has no biological activity. L-Carnitine is added to infant formulas to raise their carnitine concentration to that of human milk.

7.9.3 Pyrroloquinoline Quinone

Pyrroloquinoline quinone (PQQ) is a tricylic quinone (Figure 7.43) that functions as a coenzyme in several bacterial oxidoreductases and has been reported to be a coenzyme in mammalian lysyl oxidase and amine oxidases. However, later findings indicate that the coenzyme originally designated as PQQ in these mammalian enzymes was misidentified and is probably 6-hydroxy-dihydroxyphenylalanine quinone [60]. Although no function of PQQ is currently known in mammals, several studies have shown a very small nutritional requirement in rats and mice that appears to be associated with the formation of connective tissue and normal reproduction [81]. In spite of this demonstrated nutritional requirement, the function of PQQ is unclear. Because of the ubiquitous nature of PQQ and its synthesis by intestinal bacteria, the development of spontaneous deficiency of PQQ in rodents or humans is unlikely.

7.9.4 Coenzyme Q_{10}

Coenzyme Q_{10} (also known as ubiquinone) is a substituted quinone whose primary biochemical function involves its action as a coenzyme in the mitochondrial electron transport system [24]. The substituted quinone moiety of coenzyme Q_{10} facilitates its redox function by accommodating two sequential one-electron reductions *in vivo* (Figure 7.44). The long isoprenoid side chain provides lipid solubility and appears to serve as a membrane anchor during its redox function in mitochondria. The ubiquinol form is a potent antioxidant and serves as a component of the oxidative defense system protecting membrane lipids and, as such, it may have relevance to certain food systems.

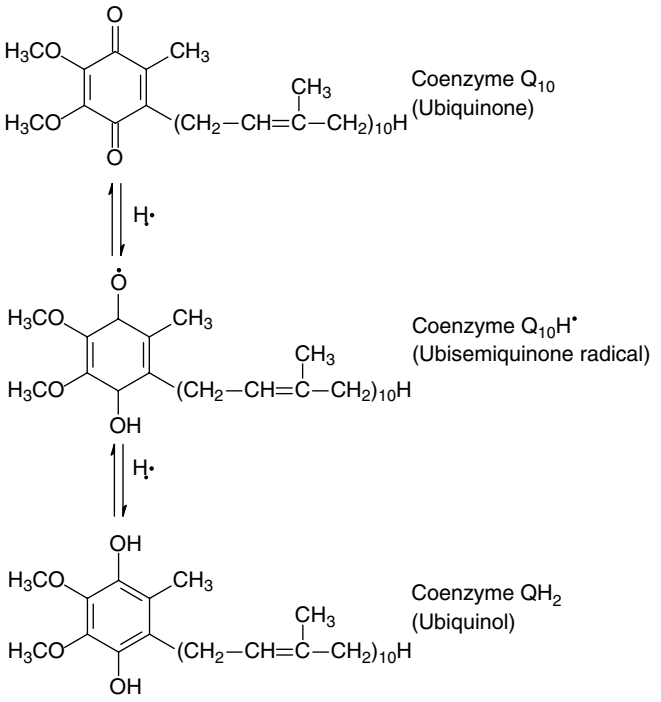


FIGURE 7.44 Structure of coenzyme Q_{10}.

Coenzyme Q_{10} is not an essential nutrient because it is synthesized in ample quantities by the human body; however, dietary sources (both plant and animal) do appear to contribute at least partially bioavailable coenzyme Q_{10} for utilization by humans. At the present time, there is little evidence that supplemental coenzyme Q_{10} is necessary or beneficial for the maintenance of health. The therapeutic administration of coenzyme Q_{10} may be useful in nutritional support in certain forms of cancer, heart disease, Parkinson's disease, to counteract antagonistic effects of certain drugs, and in certain inherited disorders of mitochondrial metabolism.

7.10 OPTIMIZATION OF VITAMIN RETENTION

To varying degrees, inevitable losses of nutritional value occur during the course of the postharvest handling, cooking, processing, and storage of foods. Such losses occur in the food processing industry, in food service establishments, and in the home. Optimization of nutrient retention is a responsibility of food manufacturers and processors and is in the mutual interest of the industry and the public. Likewise, maximization of nutrient retention in the home, and in institutional and retail food service, is an opportunity that should not be overlooked.

Many approaches to optimization of vitamin retention are based on the chemical and physical properties of the particular nutrients involved. For example, the use of acidulants, if compatible with the product, would promote the stability of thiamin and AA. Reduction in pH would decrease the stability of certain folates, however, which illustrates the complexity of this approach. Cooking or commercial processing under conditions that minimize exposure to oxygen and excess liquid lessens the oxidation of many vitamins and the extraction (i.e., leaching) of vitamins and minerals. HTST conditions will, in many instances, cause less vitamin degradation than will conventional thermal processes of equal thermal severity (based on microbial inactivation). In addition, certain combinations of ingredients can enhance retention of several nutrients (e.g., the presence of natural antioxidants would favor retention of many vitamins).

Several examples of nutrient optimization follow. The reader is referred to additional discussions of this topic [75,91].

7.10.1 Optimization of Thermal Processing Conditions

Losses of nutrients frequently occur during thermal processing procedures intended to provide a shelf-stable product. Such losses often involve both chemical degradation and leaching. The kinetics and thermodynamics of chemical changes involving the destruction of microorganisms and vitamins differ markedly. Thermal inactivation of microorganisms occurs largely by denaturation of essential macromolecules and involves large energies of activation (typically 200–600 KJ/mol). In contrast, reactions associated with the degradation of vitamins generally exhibit activation energies of 20–100 KJ/mol. Thus, rates of microbial inactivation and rates of vitamin degradation have temperature dependencies that differ significantly. Consequently, the rate of microbial inactivation increases as a function of temperature much more rapidly than does the rate of vitamin degradation. These principles of reaction kinetics and thermodynamics form the basis of enhancement of nutrient retention when HTST conditions are used. Classical studies by Teixeira et al. [142] involved a variety of thermal processing conditions, all of which provided equivalent microbial lethality. These authors showed that thiamin retention during thermal processing of pea puree could be enhanced at least 1.5-fold through selection of the proper time–temperature combination. Although many other vitamins are less labile than thiamin during the processing of low-acid foods, a similar enhancement of their retention would be predicted.

7.10.2 Prediction of Losses

Predicting the magnitude of losses of vitamins requires accurate knowledge of degradation kinetics and temperature dependence of the particular form(s) of the vitamin(s) considered in the chemical

milieu of the food(s) of interest. Different chemical forms of vitamins react differently to various food compositions and to specific processing conditions. One must first determine whether kinetic studies of *total content* (i.e., sum of all forms) of the vitamin of interest yield useful information, or whether more specific information on the various forms of the vitamin is needed [55]. Processing studies must be conducted under conditions identical to those prevailing during the actual commercial processing or storage condition being modeled because of the sensitivity of many nutrients to their chemical and physical environments. As summarized previously [67,89], reaction kinetics should be obtained at several temperatures to permit calculation of rate constants and an energy of activation. In addition, the experimental conditions should be selected to provide sufficient loss of the vitamin being studied so that the rate constant can be determined with appropriate precision [67]. Accelerated storage studies may be performed if the kinetics and mechanisms at elevated temperature are consistent with those occurring under the actual storage conditions. Because temperatures fluctuate during actual storage and transportation of foods, models of vitamin stability should include provisions for assessing the effects of temperature fluctuation [46,85].

7.10.3 Effects of Packaging

Packaging influences vitamin stability in several ways. In canning, foods that transmit heat energy primarily by conduction (solids or semisolids) will undergo greater overall loss of nutrients than will foods that transmit heat by convection, especially if large containers are used. This difference is caused by the requirement that the thermal process must be based on the "slowest to heat" portion of the product, which, for conduction-heating foods, is the geometric center of the container. Such losses are minimized by using containers with a large surface area-to-mass ratio, that is, small cans and noncylindrical containers such as pouches [118]. Pouches also offer the advantage of requiring less liquid for filling; thus, leaching of nutrients during the processing of particulate foods can be minimized.

The permeability of the packaging material also can have a substantial effect on the retention of vitamins during food storage. AA in juices and fruit beverages have been shown to exhibit much greater stability when packages with low permeability to oxygen are used [74]. In addition, use of opaque packaging materials prevents the photochemical degradation of photolabile vitamins such as vitamin A and riboflavin and of other nutrients that are susceptible to photosensitized modes of degradation.

7.11 SUMMARY

As discussed in this chapter, vitamins are organic chemicals that exhibit a wide range of properties with respect to stability, reactivity, susceptibility to environmental variables, and influence on other constituents of foods. Prediction of net vitamin retention or mechanisms of degradation under a given set of circumstances is often fraught with difficulty because of the multiple forms of most vitamins. With that caveat, the reader is referred to Table 7.1 as an overview of the general characteristics of each vitamin.

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8 Minerals

Dennis D. Miller

CONTENTS

8.1 INTRODUCTION

Ninety chemical elements occur naturally in the earth's crust. About 25 are known to be essential to life and therefore are present in living cells (Figure 8.1). Since our food is ultimately derived from living plants or animals, we can expect to find these 25 elements in foods as well. Foods also contain other elements because living systems can accumulate nonessential as well as essential elements from their environment. Moreover, elements may enter foods as contaminants during harvesting, processing, and storage or they may be present in intentional food additives.

While there is no universally accepted definition of "mineral" as it applies to food and nutrition, the term usually refers to elements other than C, H, O, and N that are present in foods. These four nonmineral elements are present primarily in organic molecules and water, and constitute about 99% of the total number of atoms in living systems [24]. Thus mineral elements are present in relatively low concentrations in foods. Nonetheless, they play key functional roles in both living systems and foods.

Historically, minerals have been classified as either major or trace, depending on their concentrations in plants and animals. This classification arose at a time when analytical methods were not capable of measuring small concentrations of elements with much precision. Thus the term "trace" was used to indicate the presence of an element that could not be measured accurately. Today, modern methods and instruments allow for extremely precise and accurate measurement of virtually all of the elements in the periodic table [75]. Nevertheless, the terms major and trace continue to be used to describe mineral elements in biological systems. Major minerals include calcium, phosphorus, magnesium, sodium, potassium, and chloride. Trace elements include iron, iodine, zinc, selenium, chromium, copper, fluorine, and tin.

I-A	II-A	III-B	IV-B	V-B	VI-B	VII-B	VIII	VIII	VIII	I-B	II-B	III-A	IV-A	V-A	VI-A	VII-A	O
H																	He
Li	Be											B	C	N	O	F	Ne
Na	Mg											Al	Si	P	S	Cl	Ar
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br	Kr
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	Xe
Cs	Ba	Ln	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At	Rn
Fr	Ra	Ac	Th	Pa	U												

FIGURE 8.1 Periodic table of the naturally occurring elements. Shaded elements are believed to be essential nutrients for animals and humans.

8.2 PRINCIPLES OF MINERAL CHEMISTRY

Mineral elements are present in foods in many different chemical forms. These forms are commonly referred to as *species* and include compounds, complexes, and free ions [109]. Given the diversity of chemical properties among the mineral elements, the number and diversity of nonmineral compounds in foods that can bind mineral elements, and the chemical changes that occur in foods during processing and storage, it is not surprising that the number of different mineral species in foods is very large. Since foods are so complex and since many mineral species are transient, it is very difficult to isolate and characterize mineral species in foods. Thus, our understanding of the exact chemical forms of minerals in foods remains limited. Fortunately, principles and concepts from the vast literature in inorganic, organic, and biochemistry can be very useful in guiding predictions about the behavior of mineral elements in foods.

8.2.1 Solubility of Minerals in Aqueous Systems

Most nutrients are delivered to and metabolized by organisms in an aqueous environment. Thus, the availabilities and reactivities of minerals depend, in large part, on their solubility in water. This excludes the elemental form of nearly all elements (dioxygen and nitrogen are exceptions) from physiological activity in living systems since these forms (e.g., elemental iron) are insoluble in water and therefore unavailable for incorporation into organisms or biological molecules.

The species (forms) of elements present in food vary considerably depending on the chemical properties of the element. Elements in groups I-A and VII-A (Figures 8.1 and 8.8) exist in foods predominantly as free ionic species (Na^+, K^+, Cl^-, and F^-). These ions are highly water soluble and have low affinities for most ligands. Thus, they exist primarily as free ions in aqueous systems. Most other minerals are present as weak coordinate complexes, chelates, or oxygen-containing anions (see below for a discussion of complexes and chelates).

The solubilities of mineral complexes and chelates may be very different from solubilities of inorganic salts. For example, if ferric chloride is dissolved in water, the iron will soon precipitate as ferric hydroxide. On the other hand, ferric iron chelated with citrate is quite soluble. Conversely, calcium as calcium chloride is very soluble while calcium chelated with oxalate ion is insoluble.

8.2.2 Minerals and Acid/Base Chemistry

Much of the chemistry of the mineral elements can be understood by applying the concepts of acid/base chemistry. Moreover, acids and bases may profoundly influence functional properties and stabilities of other food components by altering the pH of the food. Thus acid/base chemistry is critically important in food science. A brief review of acid/base chemistry follows. For a more complete treatment of this topic, see Shriver et al. [100] or other textbooks on inorganic chemistry.

8.2.2.1 Bronsted Theory of Acids and Bases

A Bronsted acid is any substance capable of donating a proton.
A Bronsted base is any substance capable of accepting a proton.

Many acids and bases occur naturally in foods and they may be used as food additives or processing aids. Common organic acids include acetic, lactic, and citric acids. Phosphoric acid is an example of a mineral acid present in foods. It is used as an acidulant and flavoring agent in some carbonated soft drinks. It is a tribasic acid (contains three available protons):

$$H_3PO_4 \leftrightarrows H_2PO_4^- + H^+, \quad pK_1 = 2.12$$

$$H_2PO_4^- \leftrightarrows HPO_4^{-2} + H^+, \quad pK_2 = 7.1$$

$$HPO_4^{-2} \leftrightarrows PO_4^{-3} + H^+, \quad pK_3 = 12.4$$

Other common mineral acids include HCl and H_2SO_4. They are not added to foods directly, although they may be generated in foods during processing or cooking. For example, H_2SO_4 is produced when the common leavening acid sodium aluminum sulfate is heated in the presence of water:

$$Na_2SO_4 \bullet Al_2(SO_4)_3 + 6H_2O \rightarrow Na_2SO_4 + 2Al(OH)_3 + 3H_2SO_4$$

8.2.2.2 Lewis Theory of Acids and Bases

An alternative, and more general, definition of an acid and a base was developed by G.N. Lewis in the 1930s [100]:

A Lewis acid is an electron pair acceptor.
A Lewis base is an electron pair donor.

By convention, Lewis acids are often represented as "A" and Lewis bases as ":B." The reaction between a Lewis acid and a Lewis base then becomes:

$$A + :B \rightarrow A - B$$

It is important to remember that the above reaction does not involve a change in the oxidation state of either A or B, that is, it is not a redox reaction. Thus, A must possess a vacant low-energy orbital and B must possess an unshared pair of electrons. The bonding results from the interaction of orbitals from the acid and the base to form new molecular orbitals. The stability of the complex depends in large part on the reduction of electronic energy that occurs when orbitals from A and :B interact to form bonding molecular orbitals. The electronic structures of these complexes are very intricate since multiple atomic orbitals may be involved. The d-block metals, for example, can contribute up to nine atomic orbitals (1s, 3p, and 5d orbitals) to the formation of molecular orbitals. The product of the reaction between a Lewis acid and a Lewis base is commonly referred to as a complex where A and :B are bonded together through the sharing of the electron pair donated by :B.

The Lewis acid/base concept is key to understanding the chemistry of minerals in foods because metal cations are Lewis acids and they bind to Lewis bases. The complexes resulting from reactions between metal cations and food molecules range from metal hydrates, to metal-containing pigments such as hemoglobin and chlorophyll, to metalloenzymes.

The number of Lewis base molecules that may bind to a single metal ion is more or less independent of the charge on the metal ion. This number, usually referred to as the coordination number, may range from 1 to 12 but is most commonly 6. For example, Fe^{3+} binds six water molecules to form hexaaquo-iron, which takes on an octahedral geometry (Figure 8.2).

The electron donating species in these complexes are commonly referred to as *ligands*. The principal electron donating atoms in ligands are oxygen, nitrogen, and sulfur. Thus many food molecules including proteins, carbohydrates, phospholipids, and organic acids are ligands for mineral

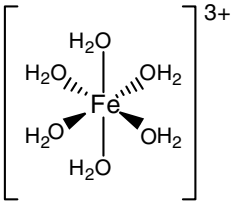


FIGURE 8.2 Ferric iron with six coordinated water molecules. This is the predominant form of Fe^{3+} in acidic (pH < 1) aqueous solutions.

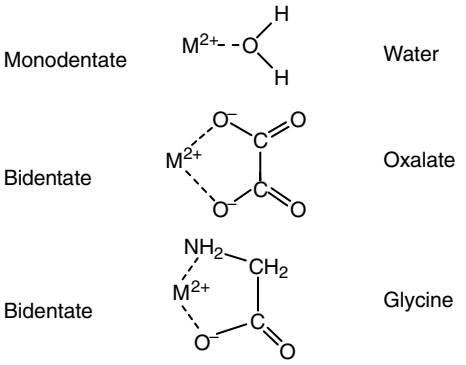


FIGURE 8.3 Examples of ligands coordinated with a metal ion (M^+).

ions. Ligands may be classified according to the number of bonds they can form with a metal ion. Those that form one bond are monodentate ligands, those that form two bonds are bidentate, and so on. Ligands that form two or more bonds are referred to collectively as multidentate ligands. Some common examples of ligands are shown in Figure 8.3.

Stabilities of metal complexes may be expressed as the equilibrium constant for the reaction representing the formation of the complex. The terms "stability constant," k, and "formation constant" are often used interchangeably. The generalized reaction for formation of a complex between a metal ion (M) and a ligand (L) is [100]

$$\begin{aligned}
&\mathrm{M} + \mathrm{L} \leftrightarrows \mathrm{ML} && k_1 = \frac{[\mathrm{ML}]}{[\mathrm{M}][\mathrm{L}]} \\
&\mathrm{ML} + \mathrm{L} \leftrightarrows \mathrm{ML}_2 && k_2 = \frac{[\mathrm{ML}_2]}{[\mathrm{ML}][\mathrm{L}]} \\
&\downarrow \downarrow \qquad \downarrow && \\
&\downarrow \downarrow \qquad \downarrow && \\
&\mathrm{ML}_{n-1} + \mathrm{L} \leftrightarrows \mathrm{ML}_n && k_n = \frac{[\mathrm{ML}_n]}{[\mathrm{ML}_{n-1}][\mathrm{L}]}
\end{aligned}$$

When more than one ligand is bound to one metal ion, the overall formation constant may be expressed as

$$K = \beta_n = \frac{[\mathrm{ML}_n]}{[\mathrm{M}][\mathrm{L}]^n}$$

where $K = \beta_n = k_1 k_2 \cdots k_n$, and n is the number of ligands bound per metal ion.

Some stability constants for Cu^{2+} and Fe^{3+} are shown in Table 8.1.

8.2.3 The Chelate Effect

A chelate is a complex resulting from the combination of a metal ion and a multidentate ligand such that the ligand forms two or more bonds with the metal resulting in a ring structure that includes the metal ion. The term chelate is derived from "chele," the Greek word for claw. Thus, a chelating ligand (also called a chelating agent) must contain at least two functional groups capable of donating electrons. In addition, these functional groups must be spatially arranged so that a ring containing the metal ion can form. Chelates have greater thermodynamic stabilities than similar complexes that are not chelates, a phenomenon referred to as the "chelate effect." Several factors interact to affect

TABLE 8.1
Stability Constants (log *K*) for Selected Metal Complexes and Chelates

Ligand	Cu^{2+}	Fe^{3+}
OH^-	6.3	11.8
Oxalate	4.8	4.8
Histidine	10.3	10.0
EDTA	18.7	25.1

Note: Values are corrected to a constant ionic strength.

Source: Adapted from Shriver, D.F., et al. (1994). *Inorganic Chemistry*, 2nd edn., W.H. Freeman, New York.

the stability of a chelate. Kratzer and Vohra [59] summarized these factors as follows:

1. *Ring size*. Five-membered unsaturated rings and six-membered saturated rings tend to be more stable than larger or smaller rings.
2. *Number of rings*. The greater the number of rings in the chelate, the greater the stability.
3. *Lewis base strength*. Stronger Lewis bases tend to form stronger chelates.
4. *Charge of ligand*. Charged ligands form more stable chelates than uncharged ligands. For example, citrate forms more stable chelates than citric acid.
5. *Chemical environment of the donating atom*. Relative strengths of metal–ligand bonds are shown below in decreasing order:
   - Oxygen as donor: $H_2O > ROH > R_2O$
   - Nitrogen as donor: $H_3N > RNH_2 > R_3N$
   - Sulfur as donor: $R_2S > RSH > H_2S$
6. *Resonance in chelate ring*. Enhanced resonance tends to increase stability.
7. *Steric hindrance*. Large bulky ligands tend to form less stable chelates.

Thus, chelate stabilities are affected by many factors and are difficult to predict. However, the concept of Gibbs free energy ($\Delta G = \Delta H - T\Delta S$) is useful for explaining the chelate effect. Consider the example of Cu^{2+} complexing with either ammonia or ethylenediamine [100]:

$$Cu(H_2O)_6^{2+} + 2NH_3 \rightarrow [Cu(H_2O)_4(NH_3)_2]^{2+} + 2H_2O$$

$$(\Delta H = -46\ \text{kJ/mol};\ \Delta S = -8.4\ \text{J/K/mol};\ \text{and}\ \log \beta = 7.7)$$

$$Cu(H_2O)_6^{2+} + NH_2CH_2CH_2NH_2 \rightarrow [Cu(H_2O)_4(NH_2CH_2CH_2NH_2)]^{2+} + 2H_2O$$

$$(\Delta H = -54\ \text{kJ/mol};\ \Delta S = +23\ \text{J/K/mol};\ \text{and}\ \log K = 10.1)$$

Both complexes have two nitrogens bound to a single copper ion (Figure 8.4) and yet the stability of the ethylenediamine complex is much greater than that of the ammonia complex (log of formation constants are 10.1 and 7.7, respectively). Both enthalpy and entropy contribute to the difference in stabilities but the entropy change is a major factor in the chelate effect. Ammonia, a monodentate ligand, forms one bond to copper while ethylenediamine, a bidentate ligand, forms two. The difference in entropy change is due to the change in the number of independent molecules in solution. In the first reaction (i.e., with NH_3), the number of molecules is equal on both sides of the equation so the entropy change is small. The chelation reaction (with ethylenediamine), on the other hand,

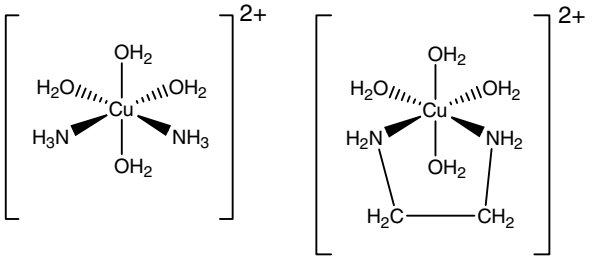


FIGURE 8.4 Cu^{2+} complexed with ammonia and ethylenediamine.

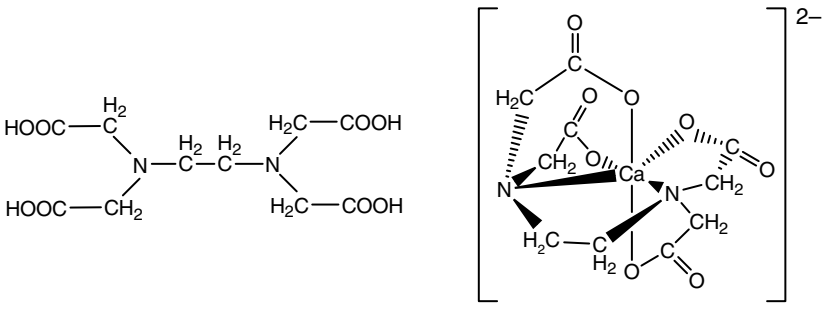


FIGURE 8.5 EDTA and a Ca^{2+}—EDTA chelate. Note that in the chelate, the carboxyl groups on EDTA are ionized, thus the net charge on the chelate is −2.

results in a net increase in the number of independent molecules in solution, and, thus, an increase in entropy.

Ethylenediaminetetraacetate ion (EDTA) provides an even more dramatic illustration of the chelate effect [85]. EDTA is a hexadentate ligand. When it forms a chelate with a metal ion in solution, it displaces six water molecules from the metal and this has a large effect on the entropy of the system (Figure 8.5):

$$Ca(H_2O)_6^{2+} + EDTA^{4-} \rightarrow Ca(EDTA)^{2-} + 6H_2O \ (\Delta S = +118 \text{ J/K/mol})$$

Moreover, EDTA chelates contain five rings, which also enhance stability. EDTA forms stable chelates with many metal ions.

Chelates are very important in foods and in all biological systems. Chelating agents may be added to foods to sequester mineral ions, such as iron or copper, to prevent them from acting as prooxidants. Preformed chelates, such as ferric sodium EDTA, may be added to foods as fortificants [10]. Furthermore, most complexes resulting from interactions between metal ions and food molecules are chelates.

8.3 NUTRITIONAL ASPECTS OF MINERALS

8.3.1 Essential Mineral Elements

Several definitions of an essential mineral element have been proposed. A widely accepted definition is the following: "An element is essential for life if its removal from the diet or other route of exposure to an organism 'results in a consistent and reproducible impairment of a physiological function'"

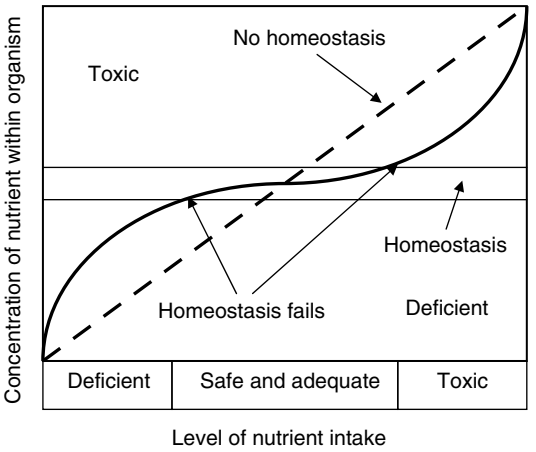


FIGURE 8.6 Homeostasis in living organisms. Without homeostasis (dashed line), the range of safe and AIs of nutrients would be very narrow. With homeostasis (solid line) the range of safe and AIs is much wider. Homeostatic mechanisms fail when intakes are very low or very high producing deficiency or toxicity, respectively. (Redrawn from Mertz, W. (1984). *Nutr Today* 19: 22–30.)

[106]. Thus, essentiality can be demonstrated by feeding diets low in a particular element to humans or experimental animals and watching for signs of impaired function.

Human requirements for essential minerals vary from a few micrograms per day up to about 1 g/day. If intakes are low for some period of time, deficiency signs will develop. Conversely, excessively high intakes can result in toxicity. Fortunately, the range of safe and adequate intake (AI) for most minerals is fairly wide so deficiency or toxicity is relatively rare provided a varied diet is consumed.

This broad range of safe and adequate intakes is possible because organisms have homeostatic mechanisms for dealing with both low and high exposures to essential nutrients. Homeostasis may be defined as the processes whereby an organism maintains tissue levels of nutrients within a narrow and constant range. In higher organisms, homeostasis is a complex set of processes involving regulation of absorption, excretion, metabolism, and storage of nutrients. Without homeostatic mechanisms, intakes of nutrients would have to be very tightly controlled to prevent deficiency or toxicity (Figure 8.6 [71]). Homeostasis can be overridden when dietary levels are excessively low or high for extended periods of time. Persistently low intakes of mineral nutrients are not uncommon, especially in poor populations where access to a variety of foods is often limited. Toxicities caused by high dietary intakes of essential minerals are less common, although high sodium intakes appear to be a major factor in hypertension (high blood pressure) [70].

Minerals are essential for hundreds of enzymatic reactions in the body, they are key players in the regulation of metabolism, they are essential for the strength and rigidity of bones and teeth, they facilitate the transport of oxygen and carbon dioxide in the blood, and they are necessary for cell adhesion and cell division. Minerals can also be toxic and there are many documented cases of severe injury and even death from exposure to minerals. Table 8.2 summarizes some of the key nutritional and toxicological aspects of minerals.

8.3.2 DRIs for Mineral Nutrients (United States and Canada)

In 1997, the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes of the Food and Nutrition Board of the Institute of Medicine issued a report describing a new approach to the establishment of appropriate dietary nutrient intakes for healthy people in the United States and

TABLE 8.2
Nutritional and Toxicological Aspects of Minerals

Mineral	Function	Deficiency Effects	Adverse Effects from Excessive Intake	Food Sources
Calcium	Bone and tooth mineralization, blood clotting, hormone secretion, nerve transmission	Increased risk for osteoporosis, hypertension, some cancers	Excessive intakes rare; may cause kidney stones, milk alkali syndrome	Milk, yogurt, cheese, fortified juices, tofu, kale, broccoli
Phosphorus	Bone mineralization; DNA and RNA synthesis; phospholipid synthesis, energy metabolism, cell signaling	Deficiency rare due to ubiquitous distribution in foods, low intakes may impair bone mineralization	Impaired bone formation, kidney stones, decreased Ca and Fe absorption, iron and zinc deficiency due to high phytate intakes	Present in virtually all foods. High protein foods (meats, dairy, etc.), cereal products, and cola beverages (as H_3PO_4) are especially rich sources
Magnesium	Cofactor for numerous enzymes	Deficiency rare except in certain clinical situations, patients recovering from cardiac surgery are often hypomagnesemic	Rarely occurs except from overconsumption of Mg supplements. Causes intestinal distress: diarrhea, cramping, nausea	Green leafy vegetables, milk, whole grains
Sodium	Predominant cation in extracellular fluid, controls extracellular fluid volume and blood pressure, required for transport of many nutrients into and out of cells	Deficiency rare except in endurance sports. Deficiency may cause muscle cramping	High intakes may lead to hypertension in salt sensitive persons	Most foods are naturally low in Na. Processed and prepared foods contain varying levels of added Na
Iron	Oxygen transport (hemoglobin and myoglobin), respiration and energy metabolism (cytochromes and iron–sulfur proteins), destruction of hydrogen peroxide (hydrogen peroxidase and catalase), and DNA synthesis (ribonucleotide reductase)	Deficiency is widespread. Effects include fatigue, anemia, impaired work capacity, impaired cognitive function, impaired immune response	Iron overload leading to increased risk for some cancers and heart disease	Meat, cereal products, fortified foods, green leafy vegetables
Zinc	Cofactor in metalloenzymes, regulation of gene expression	Growth retardation, impaired wound healing, delayed sexual maturation, impaired immune response	Inhibition of Cu and Fe absorption, impaired immune response	Red meat, shellfish, wheat germ, fortified foods
Iodine	Required for synthesis of thyroid hormones	Goiter, mental retardation, decreased fertility, cretinism	Rare in iodine replete persons, hyperthyroidism in iodine deficient persons	Iodized salt, seaweed, seafood
Selenium	Antioxidant (as component in peroxidases)	Myocarditis, osteoarthritis, increased risk for some cancers	Hair and nail loss, skin lesions, nausea	Cereals grown on high Se soils, meat from animals supplemented with Se
Lead	None, not an essential nutrient	None	Learning and behavioral problems in children, anemia, kidney damage	Contamination of foods from Pb soldered cans, exhaust from cars burning leaded gasoline, some ceramic glazes
Mercury	None, not an essential nutrient	None	Numbness, vision and hearing loss, kidney damage	Fish (especially long-lived carnivorous fish)
Cadmium	Unknown	Depressed growth in rats	Kidney damage, bone disease, cancer	Grains and vegetables grown on Cd contaminated soils

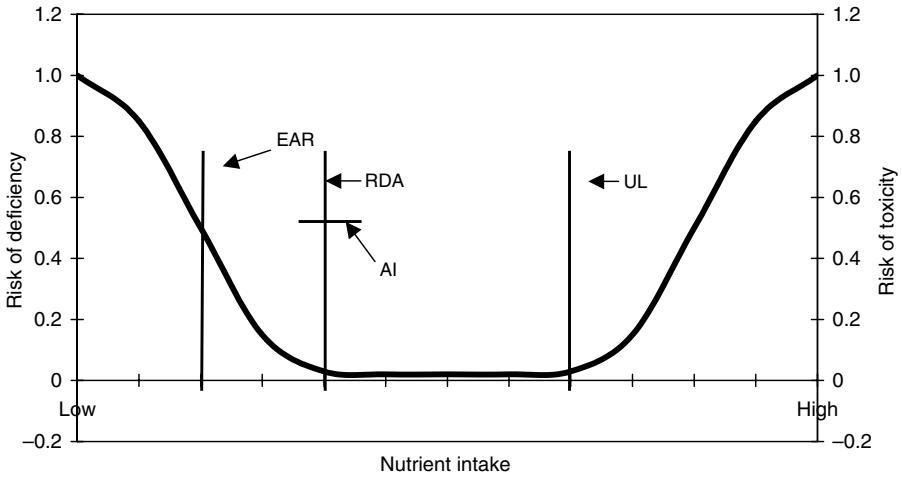


FIGURE 8.7 Risk of deficiency (left vertical axis) or excess (right vertical axis) over a range of intakes of a given nutrient for DRI categories (EAR, RDA, AI, and UL). As intakes increase, risk of deficiency decreases and approaches zero. As intakes increase beyond the safe and adequate range, risk of toxicity rises. (Redrawn from Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Food and Nutrition Board. Institute of Medicine (1997). *Dietary Reference Intakes for Calcium, Phosphorous, Vitamin D, and Flouride*. National Academy Press, Washington, D.C.)

Canada [103]. These new intake recommendations are termed "Dietary Reference Intakes" (DRIs) and replace the old Recommended Dietary Allowances (RDAs), that were first released in 1941 and have been revised periodically since that time. The last version of the RDAs was published in 1989. DRIs include a subset of values: estimated average requirement (EAR), RDA, AI, and tolerable upper intake level (UL). Each of these values is based on specific criteria used in its estimation. Brief descriptions of these are given below. For detailed descriptions, the reader is referred to the report [103].

Estimated Average Requirement. EAR is defined as the level of intake of a nutrient that meets the requirements of 50% of the individuals in a particular age and gender group. Presumably, the requirement of the remaining 50% of the individuals is higher than the EAR.

Recommended Dietary Allowance. RDA is defined as the level of intake of a nutrient sufficient to meet the requirements of nearly all healthy persons in a particular age and gender group. It is set at two standard deviations (SD) above the EAR: RDA = EAR + 2 SD.

Adequate Intake. AI is used when the available scientific evidence is insufficient to set an RDA. It is based on estimates of actual average intakes of a nutrient by healthy people, not on results from controlled studies designed to estimate individual requirements for nutrients.

Tolerable Upper Intake Level. UL is the level of intake of a nutrient below which adverse health effects are unlikely to occur. This implies that intakes above the UL may pose a risk of toxicity.

A graphical representation of EAR, RDA, AI, and UL is shown in Figure 8.7.

DRIs have been set for only 9 of the 25 minerals known to be essential for life: Ca, P, Mg, Fe, Zn, Cu, Cr, Mn, and I. The DRIs for the most important of these are listed in Tables 8.3 [39] and 8.4 [38,39].

8.3.3 Bioavailability of Minerals

It is well known that the concentration of a nutrient in a food is not necessarily a reliable indicator of the value of that food as a source of the nutrient in question. This led nutritionists to develop the

TABLE 8.3
DRIs of Nutritionally Essential Minerals (Ca, P, and Mg)[a]

	Calcium (mg/day)	Phosphorus (mg/day)	Magnesium (mg/day)
Life stage	**RDA**/AI/UL	**RDA**/AI/UL	**RDA**/AI/UL
Infants			
0–6 months	210/N.D.[b]	**100**/ND	30/N.D.
7–12 months	270/N.D.	**275**/ND	75/N.D.
Children			
1 to 3 years	500/2500	**460**/3000	**80**/65
4–8 years	800/2500	**500**/3000	**130**/110
Males			
9–13 years	1300/2500	**1250**/4000	**240**/350
14–18 years	1300/2500	**1250**/4000	**410**/350
19–30 years	1000/2500	**700**/4000	**400**/350
31–50 years	1000/2500	**700**/4000	**420**/350
50–70 years	1200/2500	**700**/4000	**400**/350
>70 years	1200/2500	**700**/3000	**400**/350
Females			
9–13 years	1300/2500	**1250**/4000	**240**/350
14–18 years	1300/2500	**1250**/4000	**360**/350
19–30 years	1000/2500	**700**/4000	**310**/350
31–50 years	1000/2500	**700**/4000	**320**/350
50–70 years	1200/2500	**700**/4000	**320**/350
>70 years	1200/2500	**700**/3000	**320**/350
Pregnancy			
≤18 years	1300/2500	**1250**/3500	**400**/350
19–30 years	1000/2500	**700**/3500	**350**/350
31–50 years	1000/2500	**700**/3500	**350**/350
Lactation			
≤18 years	1300/2500	**1250**/4000	**360**/350
19–30 years	1000/2500	**700**/4000	**310**/350
31–50 years	1000/2500	**700**/4000	**320**/350

[a] **RDAs** are printed in bold type and AI in ordinary type. The first values listed under each element are either **RDA** or AI. For example, only AIs are listed for calcium and only RDAs are listed for phosphorous while for magnesium, some are AIs, and some **RDAs**. The values listed following the forward slash (/) are the UL. In most cases, ULs are for intakes from all sources (food, water, and supplements). In the case of magnesium, however, the ULs are for intakes from supplements and do not include intakes from food and water. See text for an explanation of **RDA**, AI, and UL.

[b] N.D. = Not determined by the Food and Nutrition Board due to lack of sufficient data for making an estimate.

Source: Adapted from Food and Nutrition Board, Institute of Medicine (2003). *Dietary Reference Intake Tables: Elements Table*. (http://www.iom.edu/file.asp?id=7294)

concept of nutrient bioavailability. Bioavailability may be defined as the proportion of a nutrient in ingested food that is available for utilization in metabolic processes. In the case of mineral nutrients, bioavailability is determined primarily by the efficiency of absorption from the intestinal lumen into the blood. In some cases, however, absorbed nutrients may be in a form that cannot be utilized. For example, iron is bound so tightly in some chelates that even if the iron chelate is absorbed, the iron will not be released to cells for incorporation into iron proteins, rather the intact chelate will be excreted in the urine.

TABLE 8.4
DRIs of Nutritionally Essential Trace Minerals (Fe, Zn, Se, I, and F)[a]

	Iron (mg/day)	Zinc (mg/day)	Selenium (μg/day)	Iodine (μg/day)	Fluoride (mg/day)
Life stage	**RDA** or AI/UL	**RDA** or AI/UL	**RDA** or AI/UL	**RDA** or AI/UL	**RDA** or AI/UL
Infants					
0–6 months	0.27/40	2/4	15/45	110/N.D.[b]	0.01/0.7
7–12 months	**11**/40	**3**/5	20/60	130/N.D.	0.5/0.9
Children					
1 to 3 years	**7**/40	**3**/7	**20**/90	**90**/200	0.7/1.3
4–8 years	**10**/40	**5**/12	**30**/150	**90**/300	1/2.2
Males					
9–13 years	**8**/40	**8**/23	**40**/280	**120**/600	2/10
14–18 years	**11**/45	**11**/34	**55**/400	**150**/900	3/10
19–30 years	**8**/45	**11**/40	**55**/400	**150**/1100	4/10
31–50 years	**8**/45	**11**/40	**55**/400	**150**/1100	4/10
50–70 years	**8**/45	**11**/40	**55**/400	**150**/1100	4/10
>70 years	**8**/45	**11**/40	**55**/400	**150**/1100	4/10
Females					
9–13 years	**8**/40	**8**/23	**40**/280	**120**/600	2/10
14–18 years	**15**/45	**9**/34	**55**/400	**150**/900	3/10
19–30 years	**18**/45	**8**/40	**55**/400	**150**/1100	3/10
31–50 years	**18**/45	**8**/40	**55**/400	**150**/1100	3/10
50–70 years	**8**/45	**8**/40	**55**/400	**150**/1100	3/10
>70 years	**8**/45	**8**/40	**55**/400	**150**/1100	3/10
Pregnancy					
≤18 years	**27**/45	**12**/34	**60**/400	**220**/900	3/10
19–30 years	**27**/45	**11**/40	**60**/400	**220**/1100	3/10
31–50 years	**27**/45	**11**/40	**60**/400	**220**/1100	3/10
Lactation					
≤18years	**10**/45	**13**/34	**70**/400	**290**/900	3/10
19–30 years	**9**/45	**12**/40	**70**/400	**290**/1100	3/10
31–50 years	**9**/45	**12**/40	**70**/400	**290**/1100	3/10

[a] **RDAs** are printed in bold type and AIs in ordinary type. The first values listed under each element are either **RDA** or AI. For example, **RDAs** are listed for iron but only AIs are listed for fluoride. The values listed following the forward slash (/) are the UL. See text for an explanation of **RDA**, AI, and UL.
[b] N.D. = Not determined by the Food and Nutrition Board due to lack of sufficient data for making an estimate.

Source: Adapted from Food and Nutrition Board (FNB), Institute of Medicine (IOM). (2002). Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. National Academy Press, Washington, D.C. and Food and Nutrition Board, Institute of Medicine (2003). *Dietary Reference Intake Tables: Elements Table*. (http://www.iom.edu/file.asp?id=7294)

Bioavailabilities of mineral nutrients vary from less than 1% for some forms of iron to greater than 90% for sodium and potassium. The reasons for this wide range are varied and complex since many factors interact to determine the ultimate bioavailability of a nutrient (Table 8.5). One of the most important factors is solubility of the mineral in the contents of the small intestine since insoluble compounds cannot diffuse to the brush border membranes of enterocytes and consequently cannot be absorbed. Therefore, many of the enhancing and inhibiting factors appear to operate through effects on mineral solubility.

TABLE 8.5
Factors That May Influence Mineral Bioavailability from Foods

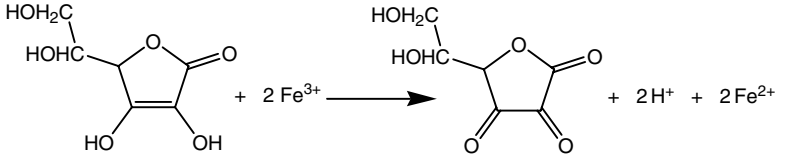
1. Chemical form of the mineral in food
   a. Highly insoluble forms are poorly absorbed
   b. Soluble chelated forms may be poorly absorbed if chelate has high stability
   c. Heme iron is absorbed more efficiently than nonheme iron in most diets
2. Food ligands
   a. Ligands that form soluble chelates with metals may enhance absorption from some foods (e.g., EDTA enhances Fe absorption from some diets)
   b. High-molecular-weight ligands that are poorly digestible may reduce absorption (e.g., dietary fiber, some proteins)
   c. Ligands that form insoluble chelates with minerals may reduce absorption (e.g., oxalate inhibits Ca absorption, phytic acid inhibits Ca, Fe, and Zn absorption)
3. Redox activity of food components
   a. Reductants (e.g., ascorbic acid) enhance absorption of iron but have little effect on other minerals
   b. Oxidants inhibit the absorption of iron
4. Mineral–mineral interactions
   a. High concentrations of one mineral in the diet may inhibit the absorption of another (e.g., Ca inhibits Fe absorption, Fe inhibits Zn absorption, Pb inhibits Fe absorption)
5. Physiological state of consumer
   a. Homeostatic regulation of minerals in the body may operate at the site of absorption, resulting in up-regulated absorption in deficiency and down-regulated absorption in adequacy or overload. This is the case for Fe, Zn, and Ca
   b. Malabsorption disorders (e.g., Crohn's disease, celiac disease) may reduce absorption of minerals and other nutrients
   c. Achlorhydria (reduced acid secretion in the stomach) may impair Fe and Ca absorption
   d. Age may affect mineral absorption: absorption efficiencies often decline with age
   e. Pregnancy: iron absorption increases during pregnancy

8.3.3.1 Bioavailability Enhancers

8.3.3.1.1 Organic Acids

Several organic acids enhance mineral bioavailability. The magnitude of the effect depends on the composition of the meal, the specific mineral nutrient, and the relative concentrations of the organic acid and the mineral. Organic acids that have received the most attention are ascorbic, citric, and lactic. Presumably, these and other organic acids improve bioavailability by forming soluble chelates with the mineral. These chelates protect the mineral from precipitation and/or binding to other ligands that may inhibit absorption.

Ascorbic acid is a particularly potent enhancer of iron absorption because, in addition to its chelating ability, it is a strong reducing agent and reduces Fe^{3+} to the more soluble and bioavailable Fe^{2+}. The following reaction shows how ascorbic acid may reduce iron [104]:



Ascorbic acid Dehydroascorbic acid

Ascorbic acid has a minimal effect on other minerals, presumably because they cannot be easily reduced.

8.3.3.1.2 Meat Factor

Meat, poultry, and fish consistently enhance the absorption of nonheme and heme iron consumed in the same meal [120]. Numerous attempts to identify and isolate the so-called "meat factor" have proven futile. Meat has a reducing effect on iron [58] so a possible mechanism is the conversion of Fe^{3+} to Fe^{2+} during digestion. In addition, products of meat digestion, including amino acids and polypeptides, may form chelates with iron that are more soluble in the contents of the small intestine.

8.3.3.2 Bioavailability Inhibitors

8.3.3.2.1 Phytic Acid

Phytic acid and various phytates are among the most important dietary factors limiting mineral bioavailability [55]. Phytic acid and its mineral complexes (phytates) are the primary storage forms of phosphorous in plant seeds. Phytic acid, *myo*-inositol-1,2,3,4,5,6-hexakisphosphate, contains six phosphate groups esterified to inositol (Figure 8.8). These phosphate groups are readily ionized at physiological pH and thus phytic acid is a potent chelator of cations, especially di- and trivalent minerals such as Ca^{+2}, Fe^{+2}, Fe^{+3}, Zn^{+2}, and Mg^{+2} (Figure 8.9 [61]). The minerals bound in these chelates may have low bioavailability, therefore, phytate is widely perceived as an antinutrient.

In addition to its well-established phosphorous storage function in plant cells, phytic acid and its derivatives serve a wide variety of roles in metabolism, including signal transduction, and possibly

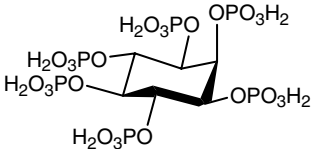


FIGURE 8.8 Chemical structure of phytic acid: *myo*-inositol-1,2,3,4,5,6-hexakisphosphate.

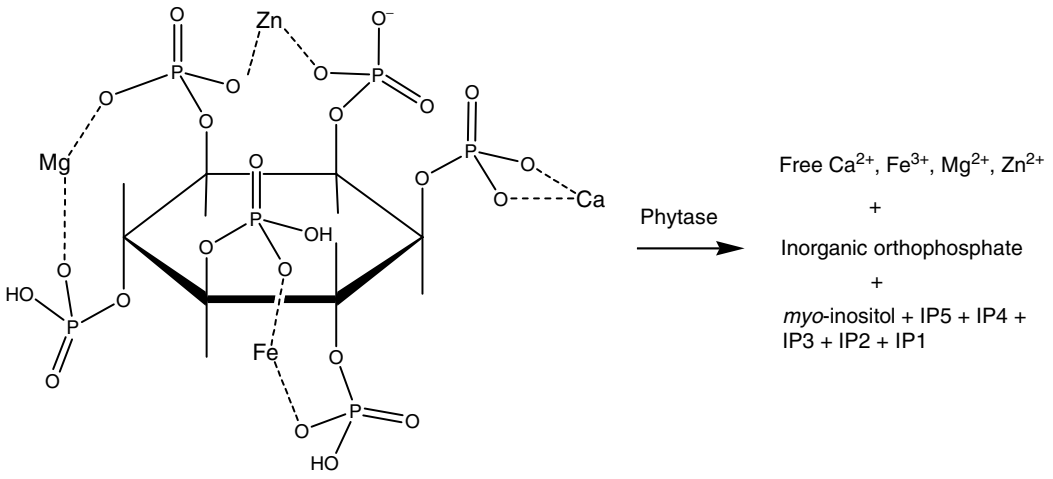


FIGURE 8.9 Haworth projection showing possible structure of a phytate-containing chelated magnesium, zinc, calcium, and iron. Ca, Mg, and Zn are divalent cations and Fe is either di- or trivalent. Phytases catalyze the hydrolysis of the phosphate groups yielding a mixture of free inositol, inositol phosphates, inorganic phosphate, and metal cations, some of which would remain bound to the partially hydrolyzed phytic acid. (Redrawn from Lei, X.G. and C.H. Stahl (2001). *Appl Microbiol Biotechnol* 57: 474–481.)

TABLE 8.6
Content of Inositol Hexakisphosphate (IP6) and Three of Its Hydrolysis Products (IP3, IP4, and IP5) in Selected Foods

Food	IP3	IP4	IP5	IP6
Bread, whole meal	0.3	0.2	0.5	3.2
Textured soy flour	—	0.9	4.4	21.8
Corn Grits, Quaker	Tr	0.03	0.3	2.0
Corn flakes, Kelloggs	Tr	0.06	0.09	0.07
Cheerios, General Mills	0.06	2.2	4.6	5.1
Oat Bran, Quaker	0.07	1.0	5.6	21.2
Oatmeal, Quaker	0.08	0.7	3.0	10.3
Rice Krispies, Kelloggs	0.05	0.4	0.9	1.2
Shredded Wheat, Nabisco	0.1	0.7	3.2	9.7
Wheaties, General Mills	0.6	1.8	3.7	5.1
All Bran, Kelloggs	0.8	3.9	11.5	22.6
Garbanzo Beans	0.1	0.56	2.04	5.18
Red Kidney Beans	0.19	1.02	2.81	9.12

Note: Values are expressed as μmol per gram of food.

Source: Adapted from Harland, B. and G. Narula (1999). *Nutr Res* 19: 947–961.

ATP, RNA export, DNA repair, and DNA recombination [89]. Phytic acid is readily hydrolyzed by enzymes known as phytases. Partial hydrolysis yields a mixture of inositol phosphates depending on the number of phosphate groups released (Figure 8.9). Phytic acid and its various hydrolysis products are commonly referred to as IP6, IP5, IP4, and so on, to indicate the number of phosphate groups esterified to the inositol moiety. The inhibitory effect of phytic acid on mineral absorption is reduced by hydrolysis but recent evidence suggests that IP5, IP4, and IP3 as well as IP6 may inhibit iron absorption [95].

Concentrations of phytates in foods vary from 1 to 3% in cereals and legumes to a fraction of 1% in roots, tubers, and vegetables [95]. Since most plants contain endogenous phytases that may be activated during food processing, prepared foods contain a mixture of inositol hexaphosphate and its various hydrolysis products. Table 8.6 lists the concentrations of these phosphates in selected foods [46]. It is apparent from comparisons of levels in whole cereal brans with refined cereals that phytates are concentrated in the bran layers of the kernel and are quite low in the endosperm. In legume seeds, on the other hand, phytate is more evenly distributed and phytate levels are high in most fractions of these seeds.

Owing to the rather consistent evidence supporting the hypothesis that phytic acid reduces the bioavailability of several essential minerals, it is reasonable to infer that reducing phytate concentrations in foods will improve mineral bioavailabilities. This has led to efforts by plant breeders to select for low phytate varieties of cereal and legume crops as a strategy for reducing the prevalence of trace mineral malnutrition [88]. In addition, soaking maize flour in water overnight to activate endogenous phytases as a strategy for reducing phytate levels has been tried in a small study in Malawi [67]. A small improvement in iron status was observed in children consuming a gruel made from the flour. Unfortunately, the effectiveness of this approach has been inconsistent and disappointing [62].

While reducing phytic acid intakes may benefit mineral nutrition status in some populations, doing so could prove to be unwise because there is compelling evidence from animal studies that phytic acid is protective against some forms of cancer [41,108]. The mechanisms involved are poorly understood but may entail antioxidant activity resulting from chelation of iron and copper.

8.3.3.2.2 Polyphenolic Compounds

Foods rich in polyphenolic compounds consistently reduce iron bioavailability from meals [119,122]. Tea is an especially potent inhibitor, presumably because of its high tannin content. Other polyphenol-rich foods that inhibit iron absorption include coffee, nonwhite beans, raisins, and sorgum [43,119].

8.3.4 Nutritional Aspects of Essential Minerals: Overview

The process of mineral nutrient digestion and absorption may be described as follows [74]. To start, the food is masticated in the mouth where salivary amylase begins the process of starch digestion. At this stage, only limited changes in mineral species occur. Next, the food is swallowed and enters the stomach where the pH is gradually lowered to about 2 by gastric acid. At this stage, dramatic changes occur in mineral species. Stabilities of complexes are changed by the altered pH and by protein denaturation and hydrolysis. Minerals may be released into solution and may re-form complexes with different ligands. In addition, transition metals such as iron may undergo a valance change when the pH is reduced. The redox behavior of iron is strongly pH dependent. At neutral pH, even in the presence of excess reducing agents like ascorbic acid, ferric iron will remain in the 3+ oxidation state. However, when the pH is lowered, ascorbic acid rapidly reduces Fe^{3+} to Fe^{2+}. Since Fe^{2+} has lower affinity than Fe^{3+} for most ligands, this reduction will promote the release of iron from complexes in food.

In the next stage of digestion, the partially digested food in the stomach is emptied into the proximal small intestine where pancreatic secretions containing sodium bicarbonate and digestive enzymes raise the pH and continue the process of protein, lipid, and starch digestion. As digestion proceeds, more new ligands are formed and existing ligands are altered in ways that undoubtedly affect their affinities for metal ions. Thus, a further reshuffling of mineral species occurs in the lumen of the small intestine resulting in a complex mixture of soluble and insoluble, and large- and small-molecular-weight species. Soluble species, including unbound mineral ions, may diffuse to the brush border surface of the intestinal epithelial cells where they may be taken by the enterocytes or pass between cells (the paracellular route). Uptake can be facilitated by a membrane carrier or ion channel, which may be an active, energy requiring process, may be saturable, and may be regulated by physiological processes.

Clearly, the process of mineral absorption and the factors that affect it are extremely complex. Moreover, changes in the speciation of minerals during digestion, although known to occur, are poorly understood. Nevertheless, results from hundreds of studies allow us to identify factors that may influence mineral bioavailability. Some of these are summarized in Table 8.5.

8.3.5 Nutritional Aspects of Essential Minerals: Individual Minerals

For various reasons, deficiencies are common for some mineral elements and rare or nonexistent for others. Moreover, there are large variations in prevalences of specific deficiencies across geographical and socioeconomic divisions. Human dietary deficiencies have been reported for calcium, cobalt (as vitamin B_{12}), chromium, iodine, iron, selenium, and zinc [48]. Calcium, chromium, iron, and zinc occur in bound forms in foods, and bioavailabilities may be low depending on the composition of the food or meal. Thus, deficiencies of these minerals result from a combination of poor bioavailability and low intakes.

Iodine is present in foods and water predominantly as the ionic, unbound form and has high bioavailability. Iodine deficiency is caused primarily by low intakes. Selenium is present in foods principally as selenomethionine but it is efficiently utilized so deficiency is caused by low intakes. Vitamin B_{12} (cobalt) deficiency is a problem only with persons on strict vegetarian diets that are low in this vitamin or in people suffering from certain malabsorption syndromes. These observations further illustrate the complexities involved in mineral bioavailability. Some bound forms of minerals have low bioavailability while other bound forms have high bioavailability. Unbound forms generally

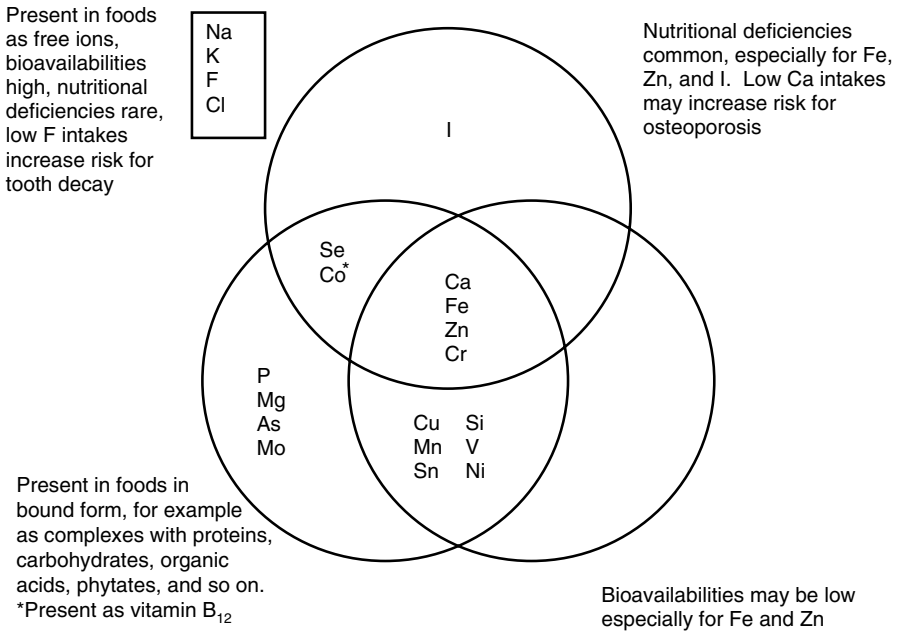


FIGURE 8.10 Essential mineral nutrients grouped according to speciation in foods (metal ions free in solution or bound in complexes or chelates), bioavailabilities, and occurrence of deficiencies in human populations.

have high bioavailability. Current thinking on bioavailability and mineral deficiencies is summarized in Figure 8.10.

In the United States, deficiencies of calcium and iron have received the most attention in recent years. In developing countries, iron and iodine have been targeted because of high prevalences of deficiencies among these populations.

8.3.5.1 Calcium

Adult male and female bodies contain approximately 1200 and 1000 g of calcium, respectively, making it the most abundant mineral in the body. More than 99% of total body calcium is present in bones [111]. Besides its structural role, calcium plays major regulatory roles in numerous biochemical and physiological processes in both plants and animals. For example, calcium is involved in photosynthesis, oxidative phosphorylation, blood clotting, muscle contraction, cell division, transmission of nerve impulses, enzyme activity, cell membrane function, intercellular adhesion, and hormone secretion.

Calcium is a divalent cation with a radius of 99 pm. Its multiple roles in living cells are related to its ability to form complexes with proteins, carbohydrates, and lipids. Calcium binding is selective. Its ability to bind to neutral oxygens, including those of alcohols and carbonyl groups, and to bind to two centers simultaneously allow it to function as a cross-linker of proteins and polysaccharides [24]).

Adequate intake levels for calcium are listed in Table 8.3. They range from 210 mg/day for infants to 1300 mg/day for adolescents and pregnant and lactating women. Calcium intakes for most population groups in the United States are well below the adequate intake level, a cause for concern. Low intakes of calcium are a factor in several chronic diseases including osteoporosis, hypertension, and some forms of cancer. However, most research on calcium and health has focused on the problem of osteoporosis. More than 10 million Americans have osteoporosis and another 34 million are at high risk for developing it [80]. Osteoporosis is a chronic disease caused by loss of bone mass. People with osteoporosis are at markedly increased risk for bone fractures, especially fractures of the hip, wrist, and vertebrae. While many factors are associated with the disease, low intakes of calcium and vitamin D appear to be among the most important.

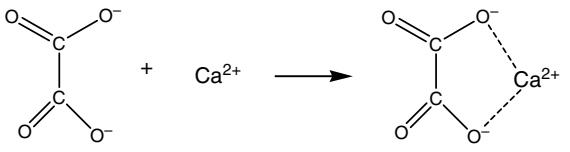


FIGURE 8.11 Formation of calcium oxalate from calcium ion and oxalate. The solubility of calcium oxalate is only 0.04 mmol/L.

TABLE 8.7
Calcium Content and Bioavailability in Selected Foods

Food	Serving Size (g)	Calcium Content (mg)	Fractional Absorption[a] (%)	Estimated Absorbable Ca/Serving (mg)	Serving to Equal 240 mL Milk (*n*)
Milk	240	300	32.1	96.3	1.0
Almonds	28	80	21.2	17.0	5.7
Pinto beans	86	44.7	17.0	7.6	12.7
Broccoli	71	35	52.6	18.4	5.2
Cabbage, green	75	25	64.9	16.2	5.9
Cauliflower	62	17	68.6	11.7	8.2
Citrus punch, with CCM[b]	240	300	50.0	150	0.64
Kale	65	47	58.8	27.6	3.5
Soy milk	120	5	31.0	1.6	6.4
Spinach	90	122	5.1	6.2	15.5
Tofu, Ca set	126	258	31.0	80.0	1.2
Turnup greens	72	99	51.6	31.1	1.9
Water cress	17	20	67.0	13.4	7.2

[a] Percent absorption adjusted for calcium load.
[b] Calcium–citrate–maleate.

Source: Weaver, C. M., and K. L. Plawecki (1994). *Am J Clin Nutr* 59(Suppl.): 1238S–1241S, Third Edition.

8.3.5.1.1 Calcium Bioavailability

The concentration of calcium in the food and the presence of inhibitors or enhancers of calcium absorption determine the absorption of calcium from foods [112]. Calcium absorption efficiency (expressed as a percentage of ingested calcium) is inversely and logarithmically related to the concentration of ingested calcium over a wide range of intakes [50]. The main dietary inhibitors of calcium absorption are oxalate and phytate with oxalate being the more potent. Calcium ions form highly insoluble chelates with oxalate (Figure 8.11). Fiber does not appear to have a major impact on calcium absorption [112].

The calcium content of several dietary sources, absorption adjusted for calcium load, and the number of servings equivalent to the absorbable calcium in one serving of milk are listed in Table 8.7. Only fortified fruit juices supply more absorbable calcium per serving than milk. These data show that it is difficult to achieve recommended intakes of calcium without consuming milk or other calcium-rich dairy products.

It is apparent from Table 8.7 that both calcium content of foods and absorbability vary widely. The percent absorption of calcium from milk is lower than that for some other foods not because it is bound in an unavailable form but because it is present at a high concentration. The poor bioavailability of

calcium from spinach and pinto beans is probably due to high concentrations of oxalate and phytate, respectively.

8.3.5.2 Phosphorus

Phosphorous is ubiquitous in all living systems owing to the vital role it plays in the structure of cell membranes and virtually all metabolic processes. It exists in soft tissues as inorganic phosphate, mostly in the form of HPO_4^{2-}, and as a constituent of numerous organic molecules. The adult human body contains up to 850 g of phosphorous of which 85% is in the skeleton in the form of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. The calcium to phosphate ratio in bone is maintained at a nearly constant mass ratio of approximately 2:1 [4].

Organic phosphates found in living systems include phospholipids, which make up the lipid bilayer in all cell membranes; DNA and RNA; ATP and creatine phosphate; cAMP (an intracellular second messenger); and many others. Thus, phosphorous is required for cell reproduction, cell integrity, transport of nutrients across membranes, energy metabolism, and regulation of metabolic processes.

RDAs for phosphorous range from 100 mg/day in infants to 1250 mg/day in adolescents and pregnant and lactating women (Table 8.3). The phosphorous RDA is very similar to the Ca adequate intake levels but, unlike the situation for Ca, P deficiency is rare except in persons with certain metabolic diseases. This is because phosphorous is so widespread in the food supply.

While phosphorous is present in virtually all foods, high protein foods such as dairy products, meat, poultry, and fish are especially rich sources. Whole grain products and legumes are also high in phosphorous but much of it is present as phytate, the primary storage form of phosphorous in seeds. Unlike inorganic phosphate and most organic phosphates, phytate phosphorous has low bioavailability and may inhibit the absorption of several trace minerals (see Section 8.3.3.2). Phosphates from food additives contribute an increasing proportion of phosphorous intakes. Phosphates are widely used in many processed foods including carbonated beverages, processed cheeses, cured meats, baked products, and many others [30].

8.3.5.3 Sodium, Potassium, and Chloride

Sodium and potassium are classified as alkali metals (group I-A of the periodic table). They readily give up their valence electron (ns^1) to form monovalent cations. They exist naturally only as salts. Sodium is the sixth most abundant element in the earth's crust. There are vast underground deposits of sodium chloride. Potassium exists naturally as KCl (sylvite) and $KCl{\bullet}MgCl_2{\bullet}6H_2O$ (carnalite). The main industrial use of potassium is in fertilizer.

Sodium, potassium, and chloride are essential nutrients but deficiencies are rare because intakes are almost always greater that the requirement. Minimum requirements are difficult to establish and RDAs/AIs have not been set. Sodium intakes vary widely across populations, ranging from approximately 1.2 to 5.9 g/day [87].

The bioavailability of sodium is very high and approximately 95% of ingested sodium is excreted in the urine. Total body sodium in a 70 kg person is approximately 100 g, 50% of which is in the extracellular fluid, 40% in bone, and 10% inside cells [87]. Sodium serves many important functions in the body. It is the major cation (as Na^+) in the extracellular fluid and it is involved in the regulation of blood pressure and the transport of many nutrients into cells. Sodium along with chloride (Cl^-) is critical for the regulation of the extracellular fluid volume [65]. The functions of Na^+ and Cl^- are closely intertwined and it is sometimes difficult to separate their roles in metabolism [87].

It is widely accepted that salt consumption is too high in many populations and that even modest reductions in salt intake would reduce deaths from stroke and coronary heart disease [49]. Many public health authorities recommend that the food processing and restaurant industries reduce the salt content of processed and prepared foods [79]. Individual consumers are encouraged to use less

salt in cooking, and to choose foods that are lower in salt. The minimum sodium intake required is approximately 500 mg/day. NaCl is 40% sodium by weight, so the 2400 mg recommended upper limit translates into approximately 6 g of salt per day. Most Americans consume substantially more than this. Estimated sodium intakes by adult males and females in the United States in 1990–1992 were 3229 and 2386 mg, respectively [32]. Approximately 75% of the salt in the American diet comes from foods that are commercially processed or prepared, making it difficult for many to reduce their sodium intakes [63].

Approximately 25% of adult Americans have high blood pressure (hypertension) and 50% have blood pressures above levels recommended for optimal health. Over half of Americans over the age of 60 have hypertension and prevalences are even higher in some ethnic groups. For example, over 80% of African-American women over the age of 60 are hypertensive [63].

8.3.5.4 Iron

Iron is the fourth most abundant element in the earth's crust and is an essential nutrient for nearly all living species. In biological systems, it is present almost exclusively as chelates with porphryrin rings or proteins. Adult male and female bodies contain approximately 4 and 2.5 g of iron, respectively. About two-thirds of this iron is functional, meaning that it plays an active role in metabolism. The remaining one-third, in iron replete individuals, is present in iron stores, located primarily in the liver, spleen, and bone marrow. Functional iron plays many key roles in biological systems, including oxygen transport (hemoglobin and myoglobin), respiration and energy metabolism (cytochromes and iron–sulfur proteins), destruction of hydrogen peroxide (hydrogen peroxidase and catalase), and DNA synthesis (ribonucleotide reductase). Many of the aforementioned proteins contain heme, a complex of iron with protoporphyrin IX (Figure 8.12). Iron's involvement in many of these metabolic reactions depends on its ability to readily accept or donate an electron (i.e., to easily redox-cycle between the Fe^{2+} and Fe^{3+} forms).

Unfortunately, free iron can be toxic to living cells. Presumably, this toxicity results from the generation of activated species of oxygen, which in turn can promote lipid oxidation or attack proteins or DNA molecules (see below). To avoid the toxic consequences of free iron, virtually all living cells have a mechanism for storing extra iron intracellularly in a nontoxic form. The iron is sequestered in the interior of a hollow protein shell called apoferritin. This protein shell is composed of 24 polypeptide subunits arranged as a sphere. Iron is deposited in the cavity of the

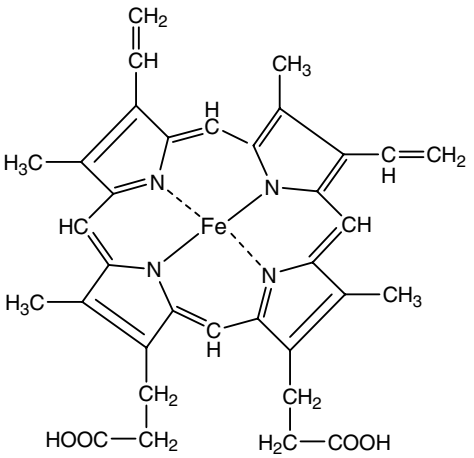


FIGURE 8.12 Heme, an iron cheletate found in many proteins including hemoglobin, myoglobin, cytochromes, and peroxidases. The oxidation state of the iron can be either II or III.

shell as polymeric ferric oxyhydroxide. Up to 4500 atoms of iron can be stored in one ferritin shell [16]. Ferritin iron is essentially a cellular reserve that can be mobilized when iron is needed for the synthesis of hemoglobin, myoglobin, or other iron proteins.

In spite of its abundance in the environment, iron deficiency in humans, some farm animals, and crops grown on some soils is a problem of major proportions. For example, Schrimshaw [99] estimated that two thirds of children and women of childbearing age in most developing countries suffer from iron deficiency. The prevalence of iron deficiency in the United States and other industrialized countries is lower than that in many developing countries but remains a persistent problem.

The paradox of high prevalences of nutritional deficiency of a nutrient present in such abundance in the environment may be explained by the behavior of iron in aqueous solutions. Iron is a transition element, which means that it has unfilled d orbitals. Its oxidation state in most natural forms is either +2 (ferrous) or +3 (ferric). Ferrous iron has six d electrons while ferric iron has five. In aqueous solutions under reducing conditions, the ferrous form predominates. Ferrous iron is quite soluble in water at physiological pHs. In the presence of molecular oxygen, however, aqueous Fe^{2+} may react with molecular oxygen to form Fe^{3+} and superoxide anion:

$$Fe^{2+}_{aq} + O_2 \rightarrow Fe^{3+}_{aq} + \bullet O_2^-$$

The hydrated Fe^{3+} will then undergo progressive hydrolysis to yield increasingly insoluble ferric hydroxide species [22]:

$$Fe(H_2O)_6^{+3} + H_2O \rightarrow Fe(H_2O)_5(OH^-)^{+2} + H_3O^+ \rightarrow\rightarrow\rightarrow Fe(OH)_3$$

Because this hydrolysis reaction occurs readily except at very low pH, the concentration of free, unbound, ferric ions in aqueous systems is vanishingly small. The predominance of low-solubility forms of iron explains why it is so poorly available.

Iron bioavailability is determined almost totally by the efficiency of iron absorption in the intestine. Total iron intake, composition of the diet, and iron status of the individual consuming the diet all play a role in determining the amount of iron absorbed.

Diets in industrialized countries like the United States consistently provide about 6 mg iron per 1000 kcal (4187 kJ) [9]. Iron species in foods may be broadly grouped as either heme or nonheme. Heme iron is firmly bound in the center of a porphyrin ring (Figure 8.12) and does not dissociate from this ligand until after it is taken up by intestinal epithelial cells. It occurs primarily as hemoglobin or myoglobin and thus is found almost exclusively in meat, poultry, and fish. Virtually all of the iron in plant foods and approximately 40–60% of the iron in animal tissues is nonheme iron. It is bound primarily to proteins but may also be complexed with citrate, phytate, oxalate, polyphenolics, or other ligands.

The bioavailability of heme iron is relatively unaffected by composition of the diet and is significantly greater than that of nonheme iron. The bioavailability of nonheme iron varies markedly depending on composition of the diet. It is widely assumed that nonheme iron from all sources in a meal (foods as well as fortification iron) enters a common pool during digestion and that absorption of iron from this pool is determined by the totality of ligands present in the digesta as it moves along the gastrointestinal tract.

Several enhancers and inhibitors of nonheme iron absorption have been identified. Enhancers include meat, poultry, fish, ascorbic acid, and EDTA (in diets where bioavailabilities are low). Inhibitors include polyphenolics (tannins in tea, legumes, and sorghum), phytates (present in legumes and whole grain cereals), some plant proteins (especially legume proteins), calcium, and phosphates.

The overall bioavailability of iron in a diet is determined by complex interactions of the enhancers and inhibitors present. Iron absorption from diets composed primarily of roots, tubers, legumes, and cereals, with limited meat and ascorbic acid, may be only about 5% even in people with poor iron status. Such a diet would provide only about 0.7 mg of absorbable iron per day, a quantity too

small to meet the needs of many individuals. Iron absorption from diets based on roots, cereals, and legumes that contain some meat, poultry, or fish and some foods high in ascorbic acid may be about 10%. These diets provide about 1.4 mg of absorbable iron per day, an amount that is adequate for most men and postmenopausal women but inadequate for up to 50% of women of childbearing age. Diets composed of generous quantities of meat, poultry, fish, and foods high in ascorbic acid provide over 2 mg of absorbable iron per day, an amount sufficient to meet the needs of nearly all healthy persons [9].

8.3.5.5 Zinc

Zinc is present in biological systems as a divalent cation, Zn^{2+}. It does not change its valence under most conditions and therefore does not participate directly in redox reactions like its sister transition elements iron and copper. It is a strong Lewis acid and therefore binds to electron-donating ligands. Ligands containing sulfhydryl groups (−SH) and amine groups bind Zn^{2+} quite strongly. Therefore, most zinc in biological systems is bound to proteins [21].

Zinc is involved in a wide range of metabolic functions. More than 50 zinc metallo enzymes have been identified. These include RNA polymerases, alkaline phosphatase, and carbonic anhydrase [21]. Zinc plays structural as well as catalytic roles in metalloenzymes. It functions as an antioxidant, presumably as a cofactor of the metalloenzyme Cu/Zn superoxidedismutase. It is also a key player in the regulation of gene expression. RDAs for zinc range from 2 mg/day in infants to 13 mg/day in teenage lactating women (Table 8.4).

Zinc deficiency in humans and animals causes an impaired immune response, delayed wound healing, and poor appetite. Prasad [86] first described clinical zinc deficiency in 1961 in boys who presented with dwarfism and hypogonadism (delayed sexual maturation). Presumably, these cases were caused by consumption of breads high in phytate [28]. Capacity for storing zinc in the body is limited, and consequently zinc deficiency can develop rapidly when intakes are low [21].

The content and bioavailability of zinc in foods varies widely. In the United States, meat and milk products are the most important sources [66,68]. Homeostatic regulation of total body zinc occurs primarily in the intestine. When intakes are low, the rate of true absorption increases and endogenous excretion of zinc via the intestine decreases [21]. Fecal excretion of endogenous zinc results from secretions in the pancreatic juice and directly through enterocytes.

Studies on the effects of phytic acid on zinc bioavailability consistently show that phytic acid impairs zinc absorption. Therefore diets rich in whole grain cereal products and legumes would be expected to increase the risk for zinc deficiency. Products made from refined flour are lower in phytic acid but are also lower in zinc since it is concentrated in the bran and germ fractions of the kernel. Sandstrom et al. [96] reported that total zinc absorption from whole wheat bread was 50% greater than from white bread even though the percentage absorptions were 17% and 38%, respectively. Nevertheless, the adequacy of vegetarian diets for zinc nutriture has been questioned. Zinc deficiency appears to be much more prevalent in developing countries compared to developed countries. In Mexico, 25% of children under the age of 11 had serum zinc levels below 10.0 μmol/L (0.65 mg/L) [94]. A possible explanation for this discrepancy is the lower consumption of meat and dairy products in developing countries. However, in developed countries such as the United States, zinc status in vegetarians does not appear to be significantly lower than in nonvegetarians although some studies have shown lower plasma zinc levels within the normal range [54]. The lack of a sensitive test for marginal zinc deficiency may be one explanation for these results.

8.3.5.6 Iodine

Iodine is an essential nutrient required for the synthesis of the thyroid hormones. These hormones, thyroxine (3,4,3′,5′ tetraiodothyronine, designated as T_4) and 3,5,3′ triiodothyronine (T_3), have multiple functions in the body [102]. They influence neuronal cell growth, physical and mental

development in children, and basal metabolic rate. RDAs for iodine range from 90 μg/day in children to 290 μg/day in lactating women (Table 8.4).

Inadequate intakes of iodine cause a variety of diseases known as iodine deficiency disorders (IDD) [25,26]. Goiter is the most widely known IDD but many other disorders may result from iodine deficiency including decreased fertility, increased rates of perinatal mortality, growth retardation in children, and impaired mental development [26]. IDD affects more than 700 million people [118]. Iodine deficiency is the leading cause of mental retardation in the world. Cretenism, its most severe form, occurs in infants whose mothers were severely iodine deficient during their pregnancy.

Iodine deficiency occurs mainly in regions where soil iodine is low due to leaching caused by melting glaciers (e.g., in mountainous regions of Bolivia), heavy rainfall, and flooding [26]. The problem of iodine deficiency may be exacerbated by the ingestion of goitrogens. Goitrogens are substances that promote goiter development. One of these is linamarin, a thioglycoside present in cassava. If it is not removed or degraded by soaking or proper cooking before the cassava in eaten, linamarin is hydrolyzed to cyanide in the intestine, absorbed, and converted to thiocyanate. Thiocyanate interferes with iodide uptake by the thyroid gland. Goitrogens are a factor in goiter development only when iodine intakes are low, as they do not cause goiter in people with adequate intakes of iodine [102].

8.3.5.7 Selenium

Selenium is an essential constituent of several important proteins in the body [20]. These include glutathione peroxidase, plasma selenoprotein P, muscle selenoprotein W, and selenoproteins found in the prostate and placenta. Glutathione peroxidase catalyzes the reduction of hydroperoxides, thereby serving an important antioxidant role. This function explains early observations that selenium could spare vitamin E in humans and animals, that is, the vitamin E requirement increases in selenium deficiency and decreases in selenium adequacy. RDAs for selenium range from 14 μg/day for infants to 70 μg/day for lactating women (Table 8.4).

Se appears in the same group in the periodic table (IV-A) as oxygen and sulfur and therefore has similar chemical properties. It is present in animal tissues primarily as selenocysteine, an amino acid with a carbon skeleton identical to serine and cysteine (Figure 8.13).

Proteins that contain Se in stoichiometric proportions are called selenoproteins. Selenocysteine is the active form of Se in proteins of animals. Selenomethionine is also present but it appears to be a storage form that occurs nonspecifically in both plants and animals as part of the methionine

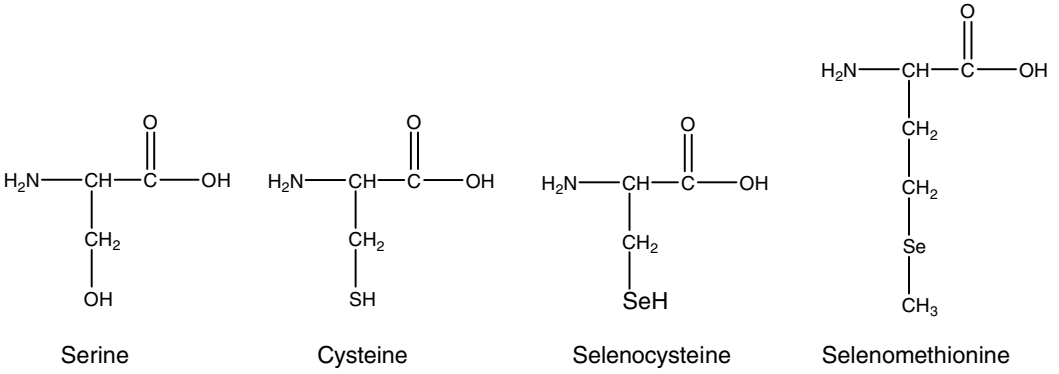


FIGURE 8.13 Chemical structures of serine, cysteine, selenocysteine, and selenomethionine. (Redrawn after from Burk, R.F. and O.A. Levander (1999). Selenium. In: *Modern Nutrition in Health and Disease*, 9th edn., M.E. Shills, J.A. Olson, M. Shike, and A.C. Ross, Lippincott Williams & Wilkens, Philadelphia, PA. pp. 265–276.)

pool [11]. Se is not known to be an essential nutrient in plants but selenomethionine is present in plant tissues at widely varying concentrations depending on the levels of bioavailable selenium in the soil where the plant was grown.

Selenium deficiency causes serious health problems in both animals and humans. Its prevalence varies widely across regions of the world. High prevalences occur in areas when soil Se levels are low and populations rely heavily on locally produced foods. Keshan disease and Kaschin-Beck disease occur in rural areas of China and eastern Siberia where soil Se levels are extremely low [20]. Keshan disease is a myocarditis (an inflammation of the middle muscular layer of the heart wall) that manifests as cardiac insufficiency, enlarged heart, heart arrhythmias, and other heart-related problems. Supplementation with sodium selenite (Na_2SeO_3) tablets have produced a dramatic reduction in the prevalence of this disease in low Se areas of China in recent years, but it is now recognized that the disease is multifactorial and possibly involves a viral infection that is more virulent in patients with Se deficiency [20]. Kaschin-Beck disease is a form of osteoarthritis that presents as joint deformities and, in severe cases, dwarfism. It has been clearly associated with Se deficiency but, as in the case of Keshan disease, other factors appear to be involved in its causation [11]. These include mycotoxins in grain and unknown organic contaminants in drinking water.

In addition to its role as a nutrient essential for preventing the aforementioned deficiency disorders, emerging evidence suggests that Se intakes above those necessary to prevent deficiencies may prevent cancer. Several epidemiological studies have found an inverse correlation between Se status and cancer. In addition, a double-blind, placebo-controlled intervention trial where the experimental group received a daily supplement of 200 μg Se showed a 37% reduction in total cancers in the supplemented group compared to the placebo group [17]. It should be noted that 200 μg is substantially higher than the RDA (55 μg for adult males).

There is also some epidemiological evidence that rates of coronary heart disease are inversely related to blood selenium levels. While this relationship is biologically plausible given that free radicals may promote the development of atherosclerosis and several selenoproteins are antioxidants, the evidence is conflicting [3].

The primary sources of Se in human diets are cereal products, meats, and seafood [2]. The concentration of Se in these foods is highly variable across regions of the world because of large variations in levels of bioavailable Se in soils. A dramatic example of this is seen in wheat grains. Wheat kernels grown in the Dakotas in the United States may contain more than 2 mg Se/kg while concentrations in wheat grown in New Zealand may be as low as 0.005 mg/kg. The Se content of animal products is also variable because levels in animal feeds that in turn are influenced by soil levels affect it. In recent decades, the practice of adding Se to animal feed supplements to prevent Se deficiency in becoming increasingly common. This practice has reduced the geographic variation in Se levels in animal products [20]. Table 8.8 lists the Se contents in selected foods available in several countries. Given the differences in Se in foods in different countries, it is no surprise that dietary Se intakes also vary across regions of the world. Table 8.9 summarizes Se intakes in several countries.

It is interesting to note that Se intakes in the United Kingdom apparently declined between 1978 and 1995. This decline has been attributed to a shift from bread wheat flour grown in the United States to wheat grown in the United Kingdom [91]. Most bread wheat grown in the United States comes from areas where soil Se levels are high.

8.3.6 Toxicology of Food-Borne Heavy Metals

All metals, including those that are essential nutrients, are toxic when intakes exceed safe levels but mercury, lead, and cadmium are most commonly considered to pose significant risks in foods.

Heavy metals find their way into foods through a variety of routes. They may be taken up from soils through the roots of plants or be deposited on the surfaces of plant leaves from airborne particulates or aerosols. Animals feeding on contaminated plants, water, or other animals may accumulate the metals in their tissues. Contaminated water may be used for irrigation, food processing, or home

TABLE 8.8
Se Content of Selected Food Categories in μg/g

		Finland[a]		China by Se Regions[b]		
Food	**USA**	**Pre-1984**	**Post-1984**	**Low Se**	**Moderate Se**	**High Se**
Cereal Products	0.06–0.66	0.005–0.12	0.01–0.27	0.005–0.02	0.017–0.11	1.06–6.9
Red meats	0.08–0.50	0.05–0.10	0.27–0.91	0.01–0.03	0.05–0.25	—
Dairy Products	0.01–0.26	0.01–0.09	0.01–0.25	0.002–0.01	0.01–0.03	—
Fish	0.13–1.48	0.18–0.98	—	0.03–0.20	0.10–0.60	—

[a] Use of Se fertilizers as a means to increase Se levels in foods was begun in Finland in 1984.
[b] China has regions of low, moderate, and high Se soils.

Source: Adapted from Combs, G.F. (2001). *Br J Nutr* 85: 517–547.

TABLE 8.9
Dietary Selenium Intakes from Selected Countries Around the World

Country or Region	**Se Intake, μg/day, Ranges**
China (low Se area)	3–11
China (high Se area)	3200–6690
Finland (1974)	25–60
Finland (1992)	90 (mean)
New Zealand	6–70
UK (1978)	60 (mean)
UK (1995)	29–39
USA	62–216

Source: Adapted from Reilly, C. (1998). *Trend Food Sci Technol* 9: 114–118.

preparation of foods. Food processing machinery and food packaging materials may contain heavy metals that leach into foods. Contamination with heavy metals may be from natural as well as man-made sources. Rain may leach heavy metals from rocks and deposit them in a bioavailable form in soils used for food production. Volcanic eruptions often contain high levels of mercury. Man-made sources include fertilizers, fungicides, sewage sludge, solder used to seal cans, clays used in the manufacture of ceramics, pigments used in paints, exhaust from automobiles burning leaded gasoline, emissions from electricity generating plants, and effluent from manufacturing plants such as paper mills. Fortunately, there has been substantial progress over the past three or four decades in reducing or eliminating contamination from many of these sources. For example, unleaded gasoline has largely replaced leaded gasoline in many countries, manufacturers have applied technologies for removing toxicants from air and water effluents, fungicides and pesticides containing mercury, and arsenic have been replaced by less toxic alternatives. Nevertheless, heavy metal contamination of foods is an ongoing concern that requires constant vigilance and monitoring.

Food-processing operations may remove heavy metal contaminants from foods as well as add them. For example, the concentration of cadmium in pasta made from durum wheat was 63% of that in the intact kernel. In contrast, lead levels in the same pasta were 120% that of the intact kernel [23].

Cooking the pasta in water reduced concentrations of cadmium and lead to 33% and 52% of the levels in the intact kernels. It should be noted that levels of both cadmium and lead in the wheat samples were well below the 0.2 μg/g (fresh weight) maximum set by the European Commission in 2001.

8.3.6.1 Lead

Lead (Pb) is a neurotoxin with potentially serious and irreversible damage to health. Children are especially vulnerable to its effects but lead poisoning also strikes adults. Signs and symptoms of lead poisoning in children include learning and behavioral problems, anemia, kidney damage, and, when exposure is high, seizures, coma, and even death [33]. The U.S. Centers for Disease Control and the World Health Organization (WHO) have declared a blood level of 10 μg/dL as a "level of concern" in children.

Fortunately, exposure to lead has dropped dramatically over the past two decades as a result of U.S. government regulations aimed at reducing lead in the environment. The use of lead in paints was banned in 1978, the addition of lead to gasoline was completely eliminated in 1995 following a 25-year phase-out program. U.S. manufacturers voluntarily discontinued the use of lead-based solder in food cans in 1991. In addition, the U.S. Food and Drug Administration (FDA) issued a regulation in 1995 banning foods packaged in cans sealed with lead-based solder thereby setting a uniform standard that applies to imported as well as domestic foods [33]. The impact of these measures has been remarkable. For example, an FDA Total Diet Study showed that daily intakes of lead from food sources by 2–5-year-olds decreased from 30 μg/day in 1982–1984 to 1.3. μg in 1994–1996. In adults, the decrease over the same period was from 38 to 2.5 μg/day.

The aforementioned measures have also yielded impressive reductions in blood levels in the U.S. population. However, scientists from the U.S. Centers for Disease and Control reported that analyses of data from the 1999–2001 National Health and Nutrition Examination Survey (NHANES) showed an estimated 434,000 children in the United States still have blood lead levels higher than 10 μg/dL [72]. Therefore, continued vigilance is required, especially in light of a recent report that blood concentrations below 10 μg/dL were shown to adversely affect IQ scores in children aged 3–5 years [12].

There is evidence from studies in experimental animals as well as in human subjects that dietary calcium competes with lead for absorption in the gastrointestinal tract. This has lead to the suggestion that persons with high calcium intakes may be partially protected from the toxic effects of lead exposure because they will absorb less of it. Presumably, the mechanism involves competition at the level of calcium binding proteins in intestinal epithelial cells for transport of the two minerals into the cell. The hypothesis is that high calcium intakes will saturate calcium-binding sites, thereby preventing or reducing binding and subsequent absorption of the lead. However, Ballew and Bowman [7] argue that the evidence supporting this hypothesis is conflicting and we should not base DRI levels for calcium on its putative protective effect on lead toxicity.

8.3.6.2 Mercury

Mercury, a toxic heavy metal, is widespread in the environment. It occurs naturally in the earth's crust and may find its way into foods through erosion and volcanic eruptions [105]. Mercury in its various forms has been used to manufacture agricultural fungicides, antibacterial drugs, thermometers, blood pressure manometers, electrical switches, and many other products. It is only relatively recently that its toxicity has become widely recognized.

8.3.6.2.1 Occurrence and Toxicity

Mercury exists in three chemical forms: elemental mercury, a liquid commonly known as quicksilver; inorganic salts of mercury; and organic mercury including phenyl and alkyl mercury compounds (e.g., methyl mercury chloride, CH_3–HgCl, and diethyl mercury, $(CH_3CH_2)_2Hg$ [1]). The Hg—Cl

bond in methyl mercury chloride is highly covalent in nature, making the compound lipophylic and therefore able to cross cell membranes [47]. Methyl mercury compounds are formed by biomethylation of inorganic mercury present in the sediment of lakes, streams, and oceans [105]. These compounds then enter the aquatic food chain and accumulate in fish and marine mammals. Concentrations are highest in long-lived predatory fish like swordfish, shark, pike, and bass [18].

The toxicity of mercury and its compounds varies with the chemical form and usually involves neurological and/or renal pathologies. Elemental mercury is poorly absorbed and readily excreted so toxic effects from oral ingestion are rare except in cases of chronic or high-level exposure [27]. However, inhalation of mercury vapors can be toxic [18] and the use of elemental mercury is being phased out in many applications including manometers used to measure blood pressure in doctors' offices. Mercury salts and organic mercury compounds, on the other hand, are highly toxic at low exposure levels. Organomercury compounds are the most toxic. Methyl mercury compounds were first synthesized in London in the 1860s and two lab technicians working on the project died from mercury poisoning [19]. A Dartmouth College chemistry professor died in 1997, 298 days after she accidentally spilled a small amount of dimethyl mercury on her gloved hand [82]. Clinical signs and symptoms involving the kidney may include glomerulonephritis and proteinuria [1]. Neurological effects can include parathaesia (numbness or tingling), ataxia (loss of coordination of voluntary muscles), neurasthenia (emotional and psychological problems), vision and hearing loss, coma, and even death [1].

Several tragic episodes of mercury poisoning resulting from food contamination have been documented. An outbreak in Minamata, Japan was caused by consumption of fish caught in Minamata bay [93]. The bay was heavily contaminated with mercury from industrial wastewater [27]. In another case in the winter of 1971–1972, an outbreak in Iraq affecting hundreds of people was caused by the mistaken use of wheat seeds treated with a fungicide-containing methyl mercury for bread baking. The seeds were meant for planting but somehow got diverted to a flourmill. There were more than 6000 cases of poisoning and 500 people died. The Environmental Protection Agency in the United States has since banned the use of alkyl mercury compounds in agriculture [1].

Now that mercury compounds have been banned from use as a fungicide, fish and marine mammals are the primary source of exposure to methyl mercury [18]. Levels of mercury in fish can vary widely as indicated in Table 8.10 [35]. While commercially caught marine fish appear to pose the greatest risk, freshwater fish may also be contaminated with mercury. The FDA has been

TABLE 8.10
Levels of Mercury in Some Species of Seafood

Species	Mean (ppm)	Range (ppm)
Tilefish	1.45	0.65–3.73
Swordfish	1.00	0.10–3.22
King Mackerel	0.73	0.30–1.67
Shark	0.96	0.05–4.54
Tuna (fresh or frozen)	0.32	N.D.–1.30
Lobster (northern, American)	0.31	0.05–1.31
Tuna (canned)	0.17	N.D.–0.75
Salmon	N.D.	N.D.–0.18
Shrimps	N.D.	N.D.

N.D. = Not detectable.

Source: Adapted from Food and Drug Administration (2001a). *Mercury Levels in Seafood Species*. http://www.cfsan.fda.gov/~acrobat/hgadv2.pdf

TABLE 8.11
Content of Cadmium in Food Categories and Estimates of Intakes in Human Populations

Food	Cd Content of the Food (mg/kg)		Typical Intake of the Food (g/day)	Exposure (μg/day)	
	Maximum	Typical		Extreme	Typical
Vegetables, including potatoes	0.1	0.05	250	25	12.5
Cereals and legumes	0.2	0.05	200	40	10
Fruit	0.05	0.01	150	7.5	1.5
Oilseeds and cocoa beans	1.0	0.5	1	1	0.5
Meat and poultry	0.1	0.02	150	15	3.0
Liver (cattle, sheep, poultry, pig)	0.5	1.0	5	2.5	0.5
Kidney (cattle, sheep, poultry, pig)	2.0	0.5	1	2	0.5
Fish	0.05	0.02	30	1.5	0.6
Crustaceans, mollusks	2	0.25	3	6	0.75
Total				93.5	30

Source: Adapted from Satarug, S., et al. (2000). *Br J Nutr* 84: 791–802.

particularly concerned about advising pregnant women to avoid consuming fish contaminated with mercury [36].

8.3.6.3 Cadmium

Chronic cadmium toxicity is associated with kidney dysfunction, bone disease, and some forms of cancer [57]. The FAO/WHO Joint Expert Committee on Food Additives (JECFA) has published a provisional tolerable weekly intake (PTWI) level of 7 μg/kg body weight per week (1 μg/kg body weight per day) for cadmium. JECFA defines PTWI as the level of intake that can be safely ingested weekly over a lifetime without significant risk for adverse health effects [97,98]. Recently, some authors have suggested that risks for kidney dysfunction increase at intakes below current PTWI levels [57,97].

Cadmium occurs naturally in soils, water, and sediments in lakes, streams, and oceans [77]. A comparison of the cadmium content of agricultural and nonagricultural soils in Australia revealed the levels in the agricultural soils was significantly higher [98]. The likely explanation is the use of cadmium contaminated phosphate fertilizers but the application of sewage sludge to soils may also be a factor. This is a concern since cadmium in soils is known to be more bioavailable to plants than lead or mercury, and food crops grown on cadmium-contaminated soils are the primary source of cadmium exposure in the general population [98].

Table 8.11 provides estimates of the cadmium content of various foods, consumption of those foods, and daily cadmium intakes. Vegetables and cereal products are the main sources of cadmium exposure. Some plants and animals are bioaccumulators of cadmium. For example, sunflower seeds typically contain higher levels of cadmium that other crops grown on the same soil. Crustaceans and mollusks are also accumulators. Fortunately, consumption of these foods is generally low. The estimated typical daily intake of cadmium is 30 μg/day which is below the 70 μg/day the FAO/WHO has set as a safe level of intake.

8.4 MINERAL COMPOSITION OF FOODS

8.4.1 Ash: Definition and Significance in Food Analysis

"Ash" is included in nutrient databases as one of the proximate components of foods. It is determined by weighing the residue left following complete combustion of the organic mater in the food and provides an estimate of the total mineral content of foods [45]. Methods for determination of ash in

specific foods and food groups are described in official publications [5]. Minerals in the ash are in the form of metal oxides, sulfates, phosphates, nitrates, chlorides, and other halides. Thus, ash content overestimates total mineral content by a considerable extent since oxygen is present in many of the anions. It does, however, provide a crude idea of mineral content and it is required for calculation of total carbohydrate in the proximate analysis scheme.

8.4.2 Individual Minerals

Individual minerals in foods are determined by ashing the food, dissolving the ash (usually in acid), and measuring mineral concentrations in the resulting solution [14,45,75]. Both chemical and instrumental methods are used to measure mineral concentrations but instrumental methods are generally more rapid, precise, and accurate. Atomic absorption spectroscopy has been available since the 1960s and is still widely used. It is a reliable technique but can measure only one mineral at a time. Inductively coupled plasma spectrometers have gained popularity in recent years primarily because they are capable of quantifying several mineral elements simultaneously from a single sample [75].

Concentrations of some minerals in selected foods are listed in Table 8.12. Sources include the United States Department of Agriculture National Nutrient Database for Standard Reference, journal articles, and manufacturer's data. Values are listed as means; data from individual foods may vary substantially from the data in Table 8.12.

8.4.3 Factors Affecting the Mineral Composition of Foods

Many factors interact to affect the mineral composition of foods so compositions can vary greatly.

8.4.3.1 Factors Affecting the Mineral Composition of Plant Foods

In order for plants to grow, they must take up water and essential mineral nutrients from the soil. Once taken up by plant roots, nutrients are transported to other parts of the plant. The ultimate composition of the edible parts of plants is influenced and controlled by genetics of the plant, fertility of the soil, and the environment in which it grows (Figure 8.14). Recent evidence suggests that the variation in the trace mineral content of cereal grains and beans is quite large (Table 8.13).

8.4.3.2 Adequacy of Plant Foods for Supplying the Mineral Needs of Humans

Plant-based foods are the primary source of nutrients for much of the world's population. Therefore it is important to understand whether plants can meet human nutrient requirements and how nutrient levels can be manipulated to enhance nutritional quality. This raises a number of questions. Do plants and humans require the same mineral nutrients? Are the concentrations of mineral nutrients in plants sufficient to meet human requirements? Can mineral concentrations in plants be altered by agricultural or genetic means to enhance the nutritional quality of plants? Are plants grown on depleted soils nutritionally inferior to plants grown on more fertile soils?

The list of essential minerals for plants is similar but not identical to the list for humans. F, Se, and I are essential for humans but not for most plants. Thus, we might expect to see human deficiencies of these elements in populations that depend on plants grown locally where soil concentrations of these elements are low. In fact, serious human deficiencies of selenium and iodine do occur in several areas of the world [20,26].

For nutrients required by both plants and animals, we might expect human deficiencies to be less of a problem because the elements will necessarily be present in plant foods. Unfortunately, concentrations of minerals in plants are sometimes too low to meet human needs, or the minerals may be present in forms that cannot be efficiently utilized by humans (see Sections 8.3.5.1 and 8.3.5.4).

TABLE 8.12
Mineral Composition of Selected Foods

Quantity	Food	Weight (g)	kcal[1]	Ca	Fe	Mg	P	K	Na	Zn	Cu	Se
1 large egg	Scrambled	61	102	43	0.73	7	104	84	171	0.61	0.009	13.7
1 slice	White bread	25	66	38	0.94	6	25	25	170	0.2	0.063	4.3
1 slice	Whole-wheat bread	28	69	30	0.68	23	57	69	132	0.5	0.106	11.3
0.5 cup	Spaghetti, cooked without salt	70	111	5	0.9	13	41	31	1	0.36	0.07	18.5
0.5 cup	Brown rice, long grain, cooked	98	108	10	0.41	42	81	42	5	0.61	0.098	9.6
0.5 cup	White rice, medium grain, cooked	93	121	3	1.39	12	34	27	0	0.39	0.035	7
0.5 cup	Black beans, cooked without salt	86	114	23	1.81	60	120	305	1	0.96	0.18	1
0.5 cup	red kidney beans, cooked without salt	89	112	25	2.6	40	126	357	2	0.95	0.214	1.1
1 cup	Whole milk	244	146	276	0.07	24	222	349	98	0.98	0.027	9
1 cup	Skim milk/nonfat milk	247	86	504	0.1	27	249	410	128	0.99	0.027	5.2
1.5 oz	American cheese, processed	42	76	287	0.18	10	347	76	601	1.39	0.014	7
1.5 cup	Cheddar cheese	113	455	815	0.77	32	579	111	702	3.51	0.035	15.7
0.5 cup	Cottage cheese, creamed, small curd	113	116	68	0.16	6	148	94	456	0.42	0.032	10.1
1 cup	Yogurt, lowfat, plain	245	154	448	0.2	42	353	573	172	2.18	0.032	8.1
0.5 cup	Ice cream, regular vanilla	72	145	92	0.06	10	76	143	58	0.5	0.017	1.3
1 each	Baked potato with skin	173	161	26	1.87	48	121	926	17	0.62	0.204	0.7
1 each	Peeled potato, boiled	167	144	13	0.52	33	67	548	8	0.45	0.279	0.5
3 each	Broccoli, raw spears	93	32	44	0.68	20	61	294	31	0.38	0.046	2.3
3 each	Broccoli spear, cooked from fresh	111	39	44	0.74	23	74	325	46	0.5	0.068	1.8
0.5 cup	Raw carrot, grated	55	23	18	0.17	7	19	176	38	0.13	0.025	0.1
0.5 cup	Cooked carrots, from frozen	73	27	26	0.39	8	23	140	43	0.26	0.06	0.4
1 each	Tomato, fresh, whole, average	123	22	12	0.33	14	30	292	6	0.21	0.073	0
0.75 cup	Tomato juice, canned	182	31	18	0.78	20	33	417	18	0.27	0.111	0.5
0.75 cup	Orange juice prepared from frozen	187	84	17	0.19	19	30	355	2	0.09	0.082	0.2
1 each	Orange, average	131	62	52	0.13	13	18	237	0	0.09	0.059	0.7
1 each	Apple with peel, average	138	72	8	0.17	7	15	148	1	0.06	0.037	0
1 each	Banana (peeled weight)	118	105	6	0.31	32	26	422	1	0.18	0.092	1.2
3 oz	Beef round, roasted	85	185	5	1.84	14	139	182	30	3.77	0.054	22.9
3 oz	Veel round, roasted	85	136	5	0.77	24	199	331	58	2.58	0.11	9.5
3 oz	Chicken, white meat, roasted	85	130	11	0.92	20	184	201	43	0.66	0.036	21.9
3 oz	Chicken, leg meat, roasted	85	151	9	1.13	17	145	190	81	1.81	0.06	16.7
3 oz	Salmon, cooked	85	127	14	0.84	28	251	352	73	0.6	0.084	48.6
3 oz	Salmon, canned, with bones	85	118	181	0.71	29	280	277	64	0.78	0.087	28.2

[1] kcal × 4.186 = kJ.

Note: Values are mg per serving, except Se is μg per serving.

Source: USDA National Nutrient Database for Standard Reference: http://www.ars.usda.gov/Services/docs.htm?docid=8964.

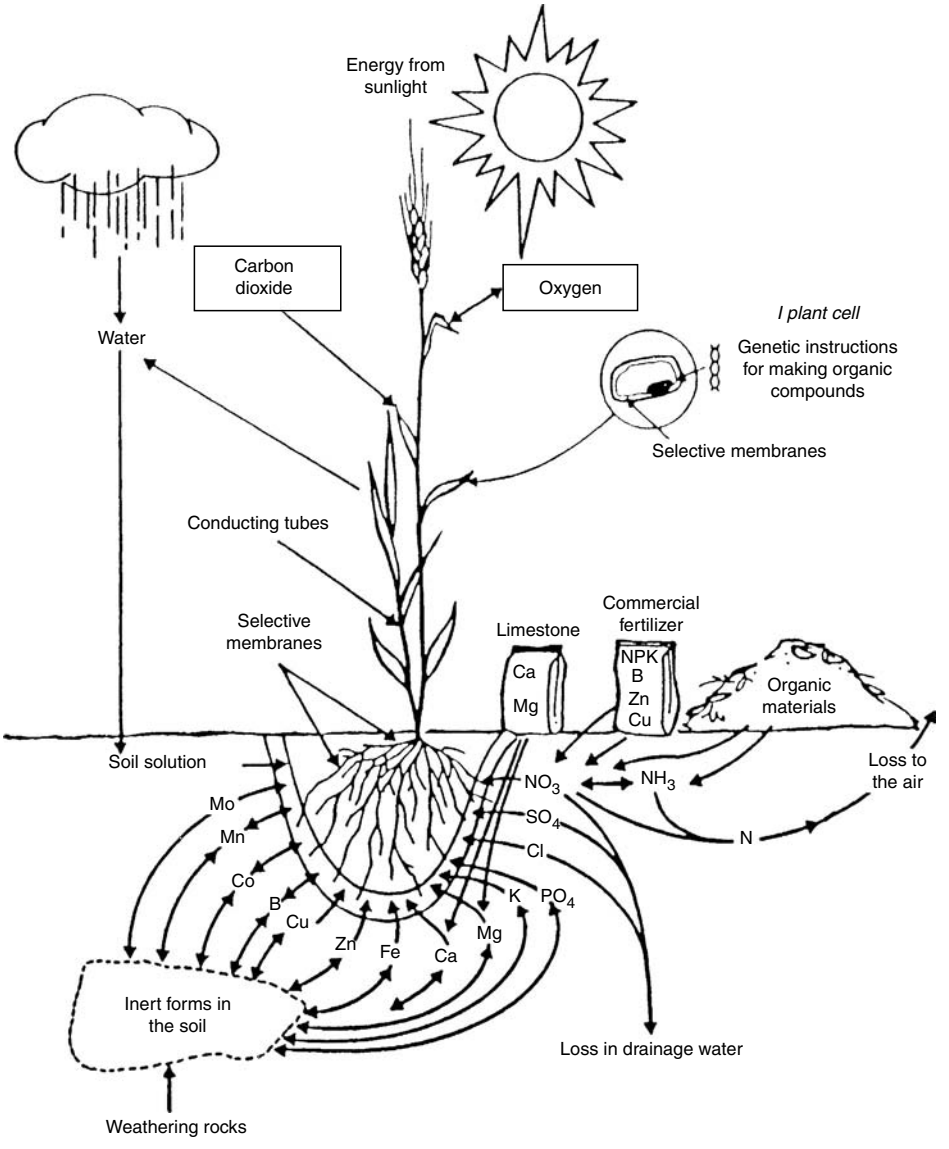


FIGURE 8.14 Plants obtain nutrients from the soil solution surrounding the roots. Sources of these nutrients include fertilizer, decaying organic matter, and weathering rocks. The minerals are taken up into the roots by a selective process and transported upward to all parts of the plant. The whole process is regulated according to instructions encoded in the plant's genome. (From Allaway, W.H. (1975). The effects of soils and fertilizers on human and animal nutrition. Agriculture Information Bulletin No. 378. U.S. Department of Agriculture. Washington, D.C.)

These situations apply, respectively, to calcium and iron. The calcium content of some plants is extremely low. Rice, for example, contains only about 10 mg calcium per 100 kcal. Thus, persons consuming rice-based diets must depend on other foods to meet calcium requirements. Iron is more uniformly distributed in plant foods than calcium but its bioavailability can be extremely poor so diets based on cereals and legumes are often inadequate in iron.

While it is possible in some cases to enhance the nutritional quality of crops through agronomic practices and plant breeding, the movement of mineral nutrients from the soil to the plant and from the plant to the animal or human is an extremely complicated process. Soils differ considerably

TABLE 8.13
Variations in the Contents of Iron and Zinc (Dry Weight Basis) in Selected Genotypes of Rice, Wheat, and Common Beans

	Fe (μ/g)		Zn (μ/g)	
Crop	**Mean**	**Range**	**Mean**	**Range**
Brown rice	13	9–23	24	13–42
Wheat	37	29–57	35	25–53
Beans	55	34–89	35	21–54

Source: Data are from Welch, R.M. and R.D. Graham (2002). *Plant Soil* 245: 205–214.

in their mineral composition. Moreover, the concentration of an element in the soil may not be a good indicator of the amount that can be taken up by plant roots since the chemical form of the element and soil pH have marked effects on mineral bioavailability to plants. For example, increasing soil pH by adding lime will lower availability of iron, zinc, manganese, and nickel to plants and will increase availability of molybdenum and selenium [114]. Also, plants generally possess physiological mechanisms for regulating amounts of nutrients taken up from the soil. Therefore, we might expect that attempts to alter the mineral composition of food crops would meet with mixed results. For example, application of fertilizer does not significantly increase iron, manganese, or calcium content of food crops [114]. On the other hand, fertilization with zinc at levels in excess of the zinc requirement of the plant has been shown to increase the level of zinc in pea seeds [115]. Moreover, there is growing evidence that genetic factors play a major role in determining mineral content of plants and that the variation among genotypes can be quite large [113]. This suggests that it should be possible to enhance the trace mineral content of these important food staples through conventional plant breeding practices, a strategy that has been termed biofortification.

8.4.3.3 Factors Affecting the Mineral Composition of Animal Foods

Mineral concentrations in animal foods vary less than mineral concentrations in plant foods. In general, changes in dietary intake of the animal have only a small effect on mineral concentrations in meat, milk, and eggs. This is because homeostatic mechanisms operating in the animal regulate tissue concentrations of essential nutrients. An exception to this is the significantly lower iron content of veal compared to beef that are range-fed or fed cereal and legume-based rations. Both are bovines but veal calves are usually fed milk-based diets that are low in iron and consequently are often iron deficient at slaughter. This can affect the iron content of the meat. For example, the iron content of braised top round of veal, separable lean only, is 1.32 mg/100 g compared to 3.32 mg/100 g for the same cut of braised beef [107].

8.4.3.4 Adequacy of Animal Foods for Supplying Mineral Needs of Humans

The composition of animal tissues is similar to that of humans, thus we might expect animal foods to be good sources of nutrients. Meat, poultry, and fish are good sources of iron, zinc, phosphate, and cobalt (as vitamin B_{12}). However, these products are not good sources of calcium unless bones are consumed, which is usually not the case. Also, the iodine content of animal foods, except marine fish, may be low. Dairy products are excellent sources of calcium. Thus, consumption of a variety of animal foods along with a variety of plant foods is the best way to ensure AIs of all essential minerals.

8.4.4 Fortification and Enrichment of Foods with Minerals

Fortification of the U.S. food supply began in 1924 with the addition of iodine to salt to prevent goiter, a prevalent public health problem in the United States at the time [13]. In the early 1940s, food fortification was expanded further when it became apparent that many young adults were failing army physical examinations owing to poor nutritional status. In 1943, the government issued an order making it mandatory to enrich flour with iron (along with riboflavin, thiamin, and niacin). Many other fortification initiatives have dealt with nutrients other than minerals, including vitamin D in 1933 and folic acid in 1998 [6].

Since the introduction of fortification back in the 1920s, there has been a dramatic reduction in the prevalences of many nutrient deficiency diseases in the United States, including iron, iodine, niacin, and vitamin D deficiencies. While general improvements in diets were major factors in this improvement in nutritional status, fortification undoubtedly deserves much of the credit for the low prevalences of nutrient deficiency diseases in the U.S. today. Rates of anemia in children in the United States have gradually declined since 1970 and they continue to fall [121]. This decline coincides with an increase in the quality and quantity of iron-fortified infant formulas and cereals, suggesting that fortification does make a difference. Another example of a successful fortification program is Chile where prevalence of iron deficiency among children has dramatically decreased following a national program to fortify milk products with iron [121].

In the U.S. today, most foods containing refined cereal grains (e.g., white flour, white rice, corn meal) are enriched with iron, niacin, riboflavin, thiamin, and folic acid. Current FDA standards for enriched flour, bread, rice, corn, and macaroni products are listed in Table 8.14 [37]. Most salt destined for domestic use is iodized. In addition, calcium, zinc, and other trace minerals are sometimes added to breakfast cereals and other products. Infant formulas contain the largest number of added minerals since they must be nutritionally complete.

8.4.4.1 Iron

In 4000 B.C., a Persian physician named Melampus made the first recorded recommendation for iron fortification [92]. He recommended that sailors consume sweet wine laced with iron filings to

TABLE 8.14
FDA Standards for the Enrichment of Cereal Products with Iron and Calcium

Food	Iron (mg/lb) (Shall Contain)	Calcium (mg/lb) (May Contain)
Enriched flour[a]	20	960
Enriched bread, rolls, and buns[a]	12.5	600
Enriched macaroni and noodle products[b]	Not less than 13	Not less than 500
	Not more than 16.5	Not more than 625
Enriched rice[b]	Not less than 13	Not less than 500
	Not more than 26	Not more than 1000
Enriched corn meals[b]	Not less than 13	Not less than 500
	Not more than 26	Not more than 750

[a] May be from any safe and suitable substance.
[b] Must be from iron and/or calcium sources that are harmless and assimilable.

Note: Products labeled as enriched must conform to these standards.

Source: Adapted from Food and Drug Administration (2003). *Code of Federal Regulations*. U.S. Government Printing Office. Chapter I, Parts 136,137,139. (http://www.gpoaccess.gov/cfr/index.html)

strengthen their resistance to spears and arrows and to enhance sexual potency. Widespread iron fortification began in the United States in 1943 when War Food Order No. 1 made enrichment of white flour sold in interstate commerce mandatory. Federal regulations no longer require flour enrichment but many state regulations do.

Addition of iron to foods is a difficult balancing act because some forms of iron catalyze oxidation of unsaturated fatty acids and vitamins A, C, and E [73]. These oxidation reactions and other interactions of the added iron with food components may produce undesirable changes in color, odor, and/or taste. In many cases, forms that are highly bioavailable are also the most active catalytically, and forms that are relatively chemically inert tend to have poor bioavailability. In general, the more water soluble the iron compound, the higher its bioavailability and the greater the tendency to adversely affect sensory properties of foods. Some commonly used iron fortificants and their properties are listed in Table 8.15 [10,34,40,53,73].

Ferrous sulfate is one of the cheapest iron sources for food fortification. It is routinely used as the reference standard in iron bioavailability studies because of its relatively high bioavailability in many foods (Table 8.15). Results of several studies have indicated that off-odors and off-flavors occur in bakery products made from flour that was heavily fortified with ferrous sulfate and stored for extended periods of time [8]. Barrett and Ranum [8] made the following recommendations for

TABLE 8.15
Characteristics of Selected Iron Fortificants Used to Fortify Food Products

Chemical Name	Formula/Formula Weight	Iron Content (g/kg Fortificant)	Solubility	Relative Bioavailability[a]
Ferrous sulfate	$FeSO_4 \cdot 7H_2O$ F.W. = 278	200	Soluble in H_2O and dilute HCl	100
Ferrous gluconate	$FeC_{12}H_{22}O_{14} \cdot H_2O$ F.W. = 482	116	Soluble in H_2O and dilute HCl	89
Ferrous fumarate	$FeC_4H_2O_4$ F.W. = 170	330	Soluble in H_2O and dilute HCl	27–200
Ferric pyrophosphate	$Fe_4(P_2O_7)_3 \cdot xH_2O$ F.W. = 745	240	Insoluble in H_2O Soluble in dilute HCl	21–74
Micronized ferric pyrophosphate	$Fe_4(P_2O_7)_3 \cdot xH_2O$ F.W. = 745	240	Water dispersible	100
Ferrous *bis*-glycinate	$FeC_4H_8O_4 \cdot H_2O$ F.W. = 240	230	Soluble in H_2O and dilute HCl	90–350
Ferric sodium EDTA	$FeNaC_{10}H_{12}N_2O_8 \cdot 3H_2O$ F.W. = 421	130	Soluble in H_2O and dilute HCl	30–390
Electrolytic iron powder	Fe F.W. = 56	970	Insoluble in H_2O, Soluble in dilute HCl	75
Hydrogen reduced iron powder	Fe F.W. = 56	97	Insoluble in H_2O, Soluble in dilute HCl	13–148
Carbonyl iron powder	Fe F.W. = 56	99	Insoluble in H_2O, Soluble in dilute HCl	5–20

[a] Relative bioavailability is compared to ferrous sulfate which is set at 100.

Sources: Adapted from Miller, D.D. (2002). In: *Nutritional Biotechnology in the Feed and Food Industries*, T.P. Lyons and K.A. Jacques, eds., Nottingham University Press, Nottingham. Additional data from Bothwell, T.H. and A.P. MacPhail (2004). *Int J Vitam Nutr Res* 74: 421–434; Fidler, M.C., et al. (2004). *Br J Nutr* 91: 107–120; *Food Chemicals Codex* (2003). 5th edn., National Academy Press, Washington, DC; and Hertrampf, E. and M. Olivares (2004). *Int J Vitam Nutr Res* 74: 435–443.

minimizing oxidation problems in bakery products that have been fortified with ferrous sulfate:

1. Ferrous sulfate is the preferred iron source for addition at the bakery.
2. Ferrous sulfate may be used to fortify wheat flour provided iron levels are kept below 40 ppm and the flour is stored at moderate temperatures and humidities for periods not exceeding 3 months.
3. Ferrous sulfate should not be used to fortify flour that may be stored for extended periods of time (as is the case with all-purpose flour intended for domestic use) or for flour that is to be used in mixes containing added fats, oils, or other easily oxidized ingredients.
4. Concentrated premixes containing ferrous sulfate and wheat flour for later addition to flour should not be used because rancidity may develop in the premix.

When fortification with ferrous sulfate is likely to cause problems in a food, other sources are commonly used. In recent years, elemental iron powders have been the sources of choice for fortification of flour for domestic use, breakfast cereals, and infant cereals. These are all products with long shelf lives.

As the name implies, elemental iron powders consist of elemental iron in a finely divided form. These forms are nearly pure iron with some contamination with other trace minerals and iron oxides. Elemental iron is insoluble in water and thus it is likely that it must be oxidized to a higher oxidation state before it can be absorbed from the intestine. Presumably, this oxidation occurs in the stomach when the iron is exposed to stomach acid:

$$Fe^0 + 2H^+ \rightarrow Fe^{2+} + H_2 \uparrow$$

Alternatively, oxygen could serve as the electron acceptor in the oxidation reaction:

$$2Fe^0 + O_2 + 4H^+ \rightarrow 2Fe^{2+} + 2H_2O$$

Reaction with oxygen could occur during food-processing operations such as bread baking. Three different types of elemental iron powders are available [83].

Reduced iron: This form is produced by reducing iron oxide with hydrogen or carbon monoxide gas and then milling to a fine powder. It is the least pure of the three types and purity depends largely on the purity of the iron oxide used [83].

Electrolytic iron: This form is produced by the electrolytic deposition of iron onto a cathode made of flexible sheets of stainless steel. The deposited iron is removed by flexing the sheets and it is then milled to a fine powder. The purity of electrolytic iron is greater than that of reduced iron. The main impurity is the iron oxide that forms on the surface during grinding and storage [83].

Carbonyl iron: This form is produced by heating scrap or reduced iron in the presence of CO under high pressure to form iron pentacarbonyl, $Fe(CO)_5$. The pentacarbonyl is then decomposed by heating to yield a very fine powder of high purity [83].

Elemental iron powders are relatively stable and do not appear to cause serious problems with oxidation in foods. However, the bioavailability of the powders is variable, probably because of differences in particle size. Iron powders are dark gray in color and may cause a slight darkening of white flour but this is not considered to be a problem [8].

Recently, there has been renewed interest in using chelated forms of iron as fortificants with sodium iron EDTA [NaFe(III)EDTA] showing considerable promise [117]. Studies in rats revealed that iron from NaFe(III)EDTA is absorbed as well or better than iron from $FeSO_4$ [29]. Numerous human trials showed that iron bioavailability from NaFe(III)EDTA in diets containing significant amounts of iron absorption inhibitors is higher than iron bioavailability from the same diets fortified

with $FeSO_4$ [10,56]. EDTA binds ferric and ferrous iron with higher affinity than other ligands such as citric acid and polyphenolic compounds [51,101]. This high affinity produces a stable chelate that may not dissociate during gastrointestinal digestion, thereby preventing the iron from binding to iron absorption inhibitors. In the absence of iron absorption inhibitors, bioavailability from NaFeEDTA may be lower than from ferrous sulfate, this explains the wide variation in relative bioavailabilities from NaFeEDTA shown in Table 8.15. In a recent prospective, double-blind, controlled study in Vietnam, Van Thuy et al. [110] showed that the prevalence of iron deficiency in women receiving fish sauce fortified with NaFeEDTA for 6 months was about 50% lower than in women in a control group who received unfortified fish sauce. A similar trial in efficacy trial in China demonstrated that NaFeEDTA-fortified soy sauce significantly reduced prevalences of iron deficiency anemia in men, women, and children [15].

Iron amino acid chelates are also promising as food fortificants [53]. The most studied of these is ferrous *bis*-glycinate, that is ferrous iron chelated with the amino acid glycine in a 1–2 molar ratio. Ferrous *bis*-glycinate is less affected by iron absorption inhibitors than ferrous sulfate. It appears to be especially effective in meals containing whole grain cereals. A major drawback to amino acid chelates is their high cost relative to ferrous sulfate or elemental iron powders [53].

8.4.4.2 Zinc

Given the apparently widespread occurrence of marginal zinc deficiency, many nutritionists advocate zinc fortification of foods as a strategy for addressing the problem. In the United States, five zinc compounds are listed as GRAS: zinc sulfate, zinc chloride, zinc gluconate, zinc oxide, and zinc stearate [94]. Of these, zinc oxide is the most commonly used for food fortification. It is more stable in foods due, in part, to its lower solubility. However, its bioavailability appears to be equal to the more soluble zinc sulfate. Fractional absorption rates of zinc from zinc oxide and zinc sulfate added to corn tortillas were 36.8 and 37.2%, respectively [94]. Moreover, zinc added as zinc sulfate to an iron fortified wheat flour dumpling decreased iron absorption in 4–8-year-old children but the same amount of zinc added as zinc oxide had no effect on iron absorption [52]. Rosado [94] recommends a fortification level of 20–50 mg Zn/kg of corn flour in Mexico.

8.4.4.3 Iodine

As mentioned above, a program for iodization of salt was adopted in the United States in 1924. In spite of the relatively simple process for adding iodine to salt and the widely recognized success of the program in the United States and other developed countries, as recently as 10 years ago salt iodization was not common in many developing countries and iodine deficiency continues to be a problem today. Fortunately, the WHO adopted an intervention strategy called Universal Salt Iodization (USI) in 1993 to tackle the problem. USI interventions strive for iodization of all salt for humans and livestock, including salt used in food processing [25]. The number of countries with a salt iodization policy increased from 43 in 1993 to 93 in 2003, and rates of goiter and mental retardation have fallen significantly as a result [118]. Unfortunately, however, IDD is still a significant problem in many areas of the world for a variety of reasons including the abundance of noniodized salt, which is cheaper and often locally produced.

Either sodium iodide (NaI) or sodium iodate ($NaIO_3$) may be used to fortify salt. Sodium iodate is often preferred because it is more stable during prolonged storage than sodium iodide, especially under conditions of high humidity and temperature [25].

8.4.5 Effects of Processing

Mineral elements, unlike vitamins and amino acids, cannot be destroyed by exposure to heat, light, oxidizing agents, extremes in pH, or other factors that affect organic nutrients. In essence, minerals are indestructible. Minerals can, however, be removed from foods by leaching or physical

TABLE 8.16
Concentrations of Selected Trace Minerals in Wheat and Milled Wheat Products

Mineral	Whole Wheat	White Flour	Wheat Germ	Millfeeds (Bran)	Loss from Wheat to Flour (%)
Iron	43	10.5	67	47–78	76
Zinc	35	8	101	54–130	78
Manganese	46	6.5	137	64–119	86
Copper	5	2	7	7–17	68
Selenium	0.6	0.5	1.1	0.5–0.8	16

Note: Values are mg mineral/kg product.

Source: Rotruck, J. T. (1982). In: *Handbook of Nutritive Value Processed Food*, M. Rechcigl, Jr., ed., CRC Press, Boca Raton, FL, Vol. I, pp. 521–528, Third Edition.

TABLE 8.17
Protein, Calcium, and Phosphate Contents of Selected Cheeses

Cheese Variety	Protein (%)	Ca (mg/100 g)	Ca:Protein (mg:g)	PO_4 (mg/100 g)	PO_4:Protein (mg:g)
Cottage	15.2	80	5.4	90	16.7
Cheddar	25.4	800	31.5	860	27.3
Emmental	27.9	920	33.1	980	29.6

Source: Guinee, T. P., P. D. Pudja, and N. Y. Farkye. (1993). In: *Cheese: Chemistry, Physics and Microbiology*, Vol. 2, 2nd edn., P. F. Fox, ed., Chapman & Hall, London, pp. 369–371 and Lucey, J. A., and P. F. Fox (1993). *J Dairy Sci* 76(6): 1714–1724, Third Edition.

separation. Also, the bioavailabilities of minerals may be altered by the factors mentioned earlier (see Section 8.3.3).

The most important factor causing mineral loss in foods is milling of cereals. Mineral elements in grain kernels tend to be concentrated in the bran layers and the germ. Removal of bran and germ leaves pure endosperm, which is mineral poor. Mineral concentrations in whole wheat, white flour, wheat bran, and wheat germ are shown in Table 8.16. Similar losses occur during milling of rice and other cereals. These are substantial losses. During fortification of milled products in the United States, iron is the only mineral commonly added.

Retention of calcium in cheese can be dramatically affected by manufacturing conditions. In cheeses where the pH is low, substantial losses of calcium occur when the whey is drained. Calcium and phosphate contents of various cheeses are shown in Table 8.17. Compositions are expressed both as mg/100 g cheese and as a Ca:protein ratio. The latter expression gives a better comparison of Ca losses because the water content of cheeses varies from one variety to another. Cottage cheese has the smallest calcium concentration because the pH at time of whey removal is typically less than 5 [44]. In Cheddar and Emmental cheeses, the whey is normally drained at pH 6.1 and 6.5, respectively. Colloidal calcium phosphate, the major fraction of Ca in milk, becomes increasingly soluble as the pH declines. Soluble Ca partitions to the whey fraction during cheese making and is lost when the whey is drained. This explains the lower Ca content in cottage cheese [64].

Since many minerals have significant solubility in water, it is reasonable to expect that cooking in water would result in some losses of minerals. Unfortunately, few controlled studies have been

done. In general, boiling in water causes greater loss of minerals from vegetables than steaming [60]. Mineral losses during cooking of pasta are minimal for iron but more than 50% for potassium [60]. This is predictable because potassium is present in foods as the free ion while iron is bound to proteins and other high- and low-molecular-weight ligands in the food.

8.5 CHEMICAL AND FUNCTIONAL PROPERTIES OF MINERALS IN FOODS

Even though minerals are present in foods at relatively low concentrations, they often have profound effects on physical and chemical properties of foods because of interactions with other food components. Details of mineral–food interactions for the broad array of minerals found in foods are given mainly in other chapters, and these interactions as well as their roles are summarized in Table 8.18. A more detailed treatment of selected minerals follows.

8.5.1 Calcium

The functional role of calcium in milk and milk products has been studied extensively and serves as an example of mineral interactions in a food system (see Chapter 14). Milk contains a complex mixture of minerals including calcium, magnesium, sodium, potassium, chloride, sulfate, and phosphate. Calcium in milk is distributed between the milk serum and the casein micelles. The calcium in serum is in solution and comprises about 30% of the total milk calcium. The remainder of the calcium is associated with casein micelles and is present primarily as colloidal calcium phosphate. It is likely that association of submicelles involves calcium bridges between phosphate groups esterified to serine residues in casein and inorganic phosphate ions.

Calcium and phosphate play an important functional role in the manufacture of cheese. Addition of calcium prior to renneting shortens coagulation time [64]. Curds with lower Ca content tend to be crumbly while cheeses higher in Ca are more elastic.

8.5.2 Phosphates

Phosphates occur in foods in many different forms, both as naturally occurring compounds intrinsic to plant and animal tissues and as components in food additives. A voluminous literature exists on the use of phosphates in foods. See Ellinger [31] and Molins [76] for in-depth treatments of this topic. Several phosphates are approved food additives. These include phosphoric acid, the orthophosphates, pyrophosphates, tripolyphosphates, and higher polyphosphates. Structures are shown in Figure 8.15.

Phosphate food additives serve many functions including acidification (soft drinks), buffering (various beverages), anticaking, leavening, stabilizing, emulsifying, water binding, and protection

TABLE 8.18
Functional Roles of Minerals and Mineral Salts/Complexes in Foods

Mineral	Food Sources	Function
Aluminum	Low and variable in foods, food additives (leavening acids, coloring agents) are major source. Endogenous Al in plant food and contamination from Al cooking vessels also contribute	*Leavening acid*: as sodium aluminum sulfate ($Na_2SO_4 \cdot Al_2(SO_4)_3$) *Colorant*: Al lakes of food dyes *Emulsifying agent*: $Na_3Al(PO_4)_2$ in processed cheese

(Continued)

TABLE 8.18 (Continued)

Mineral	Food Sources	Function
Bromine	Brominated flour	*Dough improver*: $KBrO_3$ improves baking quality of wheat flour. It has largely been replaced by ascorbic acid in the United States
Calcium	Dairy products, green leafy vegetables, tofu, fish bones, Ca-fortified foods	*Texture modifier*: forms gels with negatively charged macromolecules such as alginates, low methoxy pectins, soy proteins, caseins, etc. Increases viscosity of alginate solutions. Firms canned vegetables when added to canning brine
Copper	Organ meats, seafoods, nuts, seeds	*Catalyst*: lipid peroxidation, ascorbic acid oxidation, nonenzymatic oxidative browning *Color modifier*: may cause black discoloration in canned, cured meats *Enzyme cofactor*: polyphenoloxidase *Texture stabilizer*: stabilizes egg white foams
Iodine	Iodized salt, seafood, plants, and animals grown in areas where soil iodine is not depleted	*Dough improver*: KIO_3 improves baking quality of wheat flour
Iron	Cereals, legumes, meat, contamination from iron utensils and soil, enriched products	*Catalyst*: $Fe^{2+}$and Fe^{3+} catalyze lipid peroxidation in foods *Color modifier*: color of fresh meat depends on valence of Fe in myoglobin and hemoglobin: $Fe^{2+}$is red, Fe^{3+} is brown. Forms green, blue, or black complexes with polyphenolic compounds. Reacts with S^{2-} to form black FeS in canned foods *Enzyme cofactor*: lipoxygenase, cytochromes, ribonucleotide reductase, and so on
Magnesium	Whole grains, nuts, legumes, green leafy vegetables	*Color modifier*: removal of Mg from chlorophyll changes color from green to olive-brown
Manganese	Whole grains, fruits, vegetables	*Enzyme cofactor*: pyruvate carboxylase, superoxide dismutase
Nickel	Plant foods	*Catalyst*: hydrogenation of vegetable oils—finely divided, elemental Ni is the most widely used catalyst for this process
Phosphates	Ubiquitous, animal products tend to be good sources. Widely used food additive	*Acidulent*: H_3PO_4 in soft drinks Leavening acid: $Ca(H_2PO_4)_2$ is a fast-acting leavening acid *Moisture retention in meats*: sodium tripolyphosphate improves moisture retention in cured meats *Emulsification aid*: phosphates are used to aid emulsification in comminuted meats and in process cheeses
Potassium	Fruits, vegetables, meats	*Salt substitute*: KCl may be used as a salt substitute. May cause bitter flavor *Leavening acid*: potassium acid tartrate
Selenium	Seafood, organ meats, cereals (levels vary depending on soil levels)	*Enzyme cofactor*: glutathione peroxidase
Sodium	NaCl, MSG, other food additives, milk. Low in most raw foods.	*Flavor modifier*: NaCl elicits the classic salty taste in foods *Preservative*: NaCl may be used to lower water activity in foods *Leavening agents*: many leaving agents are sodium salts (e.g., sodium bicarbonate, sodium aluminum sulfate, sodium acid pyrophosphate)
Sulfur	Widely distributed as component of sulfur-containing amino acids, food additives (sulfites, SO_2)	*Browning inhibitor*: sulfur dioxide and sulfites inhibit both enzymatic and nonenzymatic browning. Widely used in dried fruits *Antimicrobial*: prevents, controls microbial growth. Widely used in wine making
Zinc	Meats, cereals, fortified foods	ZnO is used in the lining of cans for proteinaceous foods to lessen formation of black FeS during heating

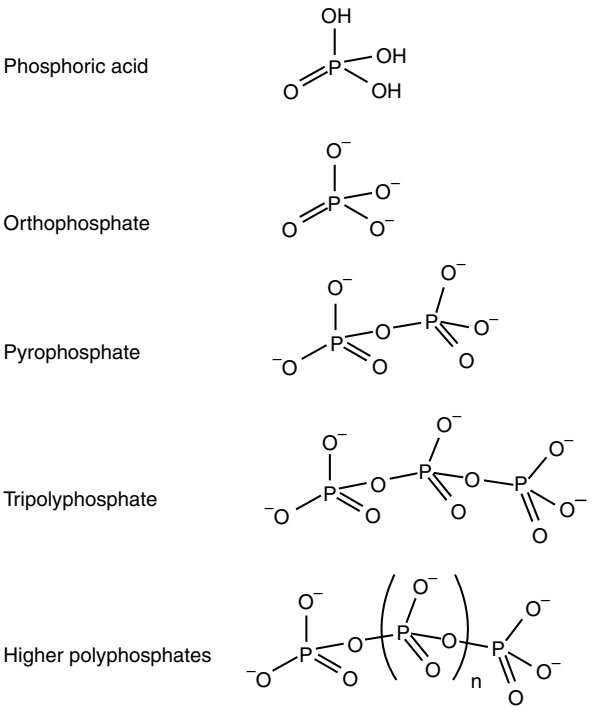


FIGURE 8.15 Structures of phosphoric acid and phosphate ions important in foods.

against oxidation. The chemistry responsible for the wide array of functional properties of phosphates is not fully understood but undoubtedly is related to the acidity of protons associated with phosphates and the charge on phosphate ions. At pHs common in foods, phosphates carry negative charges and polyphosphates behave as polyelectrolytes. These negative charges give phosphates strong Lewis-base character and thus strong tendencies to bind metal cations. An ability to bind metal ions may underlie several of the functional properties noted above. It should be mentioned, however, that there is considerable controversy about mechanisms of phosphate functionality, particularly as it relates to enhanced water-holding capacity in meats and fish.

8.5.3 Sodium Chloride

Sodium chloride (salt) is a widely used food additive. Its beneficial functions in food include enhanced flavor, control of microbial growth, improved water holding capacity in meats, and enhanced color. Salt not only adds flavor as a single ingredient but it enhances other flavors in foods and reduces bitterness. Many foods with added salt (e.g., breads and other cereal products) do not taste salty and therefore it is difficult for consumers to judge the salt content of foods based on taste. Sources of sodium in the United States food supply are listed in Table 8.19.

Salt is an essential ingredient in most cheeses. It adds to the flavor, helps to control the growth of undesirable bacteria by lowering water activity, controls the rate of lactic acid fermentation, and modifies the texture [90].

In processed meats such as sausages, salt functions as a preservative by lowering water activity. It also promotes the solubilization of muscle proteins (a salting-in phenomenon) that then function as emulsifying agents [42].

In bakery products, salt enhances flavor without imparting a salty taste, controls the rate of fermentation in yeast-leavened products, and functions as a dough improver through its interactions with gluten proteins [90].

TABLE 8.19
Contributions of Food Groups to Sodium Intakes in the U.S. Population

Food Category	Contribution to Na Intakes (Percentage of Total Intake)
Milk and milk products	6.5
Grain products	22.0
Fruits and vegetables	6.6
Meat, fish, poultry, and eggs	26.1
Mixed dishes (casseroles, soups, etc)	22.1
Fats, oils, and sauces	8.2
Deserts and sweets	4.8
Other	3.8

Adapted from Engstrom, A., et al. (1997). *Am J Clin Nutr* 65 (Suppl): 704S–707S.

8.5.4 Iron

It is well established that iron can promote lipid peroxidation in foods. Iron appears to catalyze both the initiation and propagation stages of lipid peroxidation. The chemistry is exceedingly complex but several probable mechanisms have been suggested. In the presence of reducing agents such as thiol groups and ascorbic acid, ferric iron promotes the formation of the superoxide anion [118]:

$$Fe^{3+} + RSH \rightarrow Fe^{2+} + RS\bullet + H^{+}$$

$$RSH + RS\bullet + O_2 \rightarrow RSSR + H^{+} + \bullet O_2^{-}$$

The superoxide anion may then react with protons to form hydrogen peroxide or reduce ferric iron to the ferrous form:

$$2H^{+} + 2\bullet O_2^{-} \rightarrow H_2O_2 + O_2$$

$$Fe^{3+} + \bullet O_2^{-} \rightarrow Fe^{2+} + O_2$$

Ferrous ion promotes decomposition of hydrogen peroxide to hydroxyl radicals by the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + \bullet OH$$

The hydroxyl radical is highly reactive and may rapidly generate lipid free radicals by abstracting hydrogen atoms from unsaturated fatty acids. This initiates the lipid peroxidation chain reaction.

Iron can also catalyze lipid peroxidation by accelerating decomposition of lipid hydroperoxides present in foods:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\bullet + OH^{-}$$

or

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\bullet + H^{+}$$

The rate of the first reaction is greater than the second by an order of magnitude. This explains why ascorbic acid may function as a prooxidant in some food systems since it can reduce ferric iron to the ferrous form.

8.5.5 Nickel

While nickel deficiency has never been documented in humans, there is substantial evidence of its essentiality in several animal species [81]. There is no RDA or AI for nickel. Food sources include chocolate, nuts, beans, and grains [81]. The primary significance of nickel from a food processing perspective is its use as a catalyst for the hydrogenation of edible oils [78] (see Chapter 4).

8.5.6 Copper

Copper, like iron, is a transition element and exists in foods in two oxidation states, Cu^{1+} and Cu^{2+}. It is a cofactor in many enzymes including phenolase and is at the active center of hemocyanin, an oxygen carrying protein in some arthropods. Both Cu^{1+} and Cu^{2+} bind tightly to organic molecules and thus exist primarily as complexes and chelates in foods. On the negative side, copper is a potent catalyst of lipid oxidation in foods.

An intriguing functional role of copper has been exploited in Western cuisine for at least 300 years [69]. Many recipes for meringues specify copper bowls as the preferred vessel for whipping egg whites. A common problem with egg white foams is collapse resulting from over whipping. Presumably, foam stability is reduced when the proteins at the air–liquid interface are excessively denatured by whipping. Egg white contains conalbumin, a protein analogous to the plasma iron-binding protein transferrin. Conalbumin binds Cu^{2+} as well as Fe^{3+} and the presence of bound copper or iron stabilizes conalbumin against excessive denaturation [84].

8.6 SUMMARY

Minerals are present in foods at low but variable concentrations and in multiple chemical forms. These species undergo complex changes during processing, storage, and digestion of foods. With the exception of group I-A and VII-A elements, minerals exist in foods as complexes, chelates or oxyanions. While understanding of the chemical forms and properties of many of these mineral species remains limited, their behavior in foods often can be predicted by applying principles of inorganic, organic, physical, and biological chemistry.

The primary role of minerals in foods is to provide a reliable source of essential nutrients in a balanced and bioavailable form. In cases where concentrations and/or bioavailabilities in the food supply are low, fortification has been used to help assure adequate intake by all segments of the population. Fortification with iron and iodine has dramatically reduced deficiency diseases associated with these nutrients in the United States and other industrialized countries. Unfortunately, it has not been possible to fortify appropriate staple foods in many developing countries leaving hundreds of millions of people in these countries to suffer the tragic consequences of iron, iodine, zinc, and other deficiencies.

Minerals also play key functional roles in foods. For example, minerals may dramatically alter the color, texture, flavor, and stability of foods. Thus, minerals may be added or removed from foods to achieve a particular functional effect. When manipulation of concentrations of minerals in foods is not practical, chelating agents such as EDTA (when allowed) can be used to alter their behavior.

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9 Colorants

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CONTENTS

9.1 INTRODUCTION

To understand colorants in foods some terms need to be defined. Color refers to human perception of colored materials—red, green, blue, and so forth. A colorant is any chemical, either natural or synthetic, that imparts color. Foods have color because of their ability to reflect or emit different quantities of energy at wavelengths able to stimulate the retina in the eye. The energy range to which the eye is sensitive is referred to as visible light. Visible light, depending on an individual's sensitivity, encompasses wavelengths of approximately 380–770 nm. This range makes up a very small portion of the electromagnetic spectrum (Figure 9.1). In addition to obvious colors (hues), black, white, and intermediate grays are also regarded as colors.

Pigments are natural substances in cells and tissues of plants and animals that impart color. Dyes are any substances that lend color to materials. The term dye is commonly used in the textile industry. In the U.S. food industry, a dye is a food-grade water-soluble colorant certified by the U.S. Food and Drug Administration (FDA). These specific dyes are referred to as "certified colors," and each one is assigned a food, drug and cosmetic (FD&C) number. The FD&C designation means that the dye may be used in foods, drugs, and cosmetics. Added to the approved list of certified colors are the FD&C lakes. Lakes are dyes extended on a substratum and they are oil dispersible. The dye/substratum combination is achieved by adsorption, coprecipitation, or chemical reaction. The complex involves a salt of a water-soluble primary dye and an approved insoluble base stratum. Alumina is the only approved substratum for preparing FD&C lakes. In addition, there are other dyes or lakes approved for use in other countries, where specifications are established by the European Economic Community (EEC) or the World Health Organization (WHO). Colorants exempt from certification may also be used. These are natural pigments or substances synthesized, but identical to the natural pigment. A classification of colorants and an example within each class are given in Table 9.1.

Color and appearance are major, if not the most important, quality attributes of foods. It is because of our ability to easily perceive these factors that they are the first to be evaluated by the consumer when purchasing foods. One can provide consumers the most nutritious, safest, and most economical foods, but if they are not attractive, purchase will not occur. The consumer also relates specific colors of foods to quality. Specific colors of fruits are often associated with maturity—while redness of raw meat is associated with freshness, a green apple may be judged immature (although some are green when ripe), and brownish-red meat as not fresh.

Color also influences flavor perception. The consumer expects red drinks to be strawberry, raspberry, or cherry flavored, yellow to be lemon, and green to be lime flavored. The impact of color on sweetness perception has also been demonstrated.

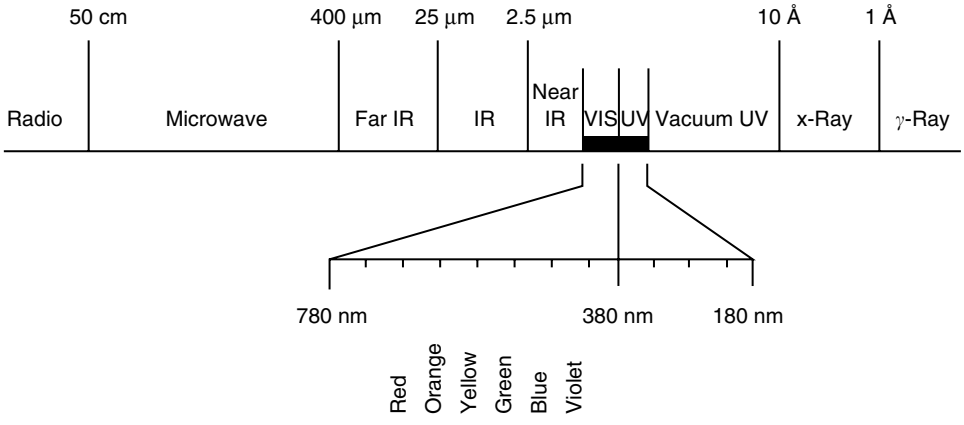


FIGURE 9.1 Electromagnetic spectrum.

TABLE 9.1
Classification of Colorants

Colorant	Example
A Certified	
1. Dye	FD&C Red No. 40
2. Lake	Lake of FD&C Red No. 40
B Exempt from certification	
1. Natural pigments	Anthocyanin, juice concentrate, annatto extract
2. Synthetic (nature identical)	β-Carotene

It should also be noted that many compounds responsible for the brilliant colors of fruits and vegetables exhibit antioxidant activity. It is clear, therefore, that color of foods has multiple effects on consumers, and it is wrong to regard color as being purely cosmetic.

Many food pigments are, unfortunately, unstable during processing and storage. Prevention of undesirable changes is usually difficult or impossible. Depending on the pigment, stability is impacted by factors such as the presence or absence of light, oxygen, metals, and oxidizing or reducing agents; temperature and water activity; and pH. Because of the instability of pigments, colorants are sometimes added to foods [31,77].

The purpose of this chapter is to provide an understanding of colorant chemistry—an essential prerequisite to manage the color and color stability of foods.

9.2 PIGMENTS IN ANIMAL AND PLANT TISSUE

Naturally occurring pigments in plants and animal tissues are those that are synthesized and accumulated or excreted from living cells. In addition, transformations occurring in foods during processing may result in formation or transformation of those colors. Pigments indigenous to animals and plants have always formed as a part of the normal human's diet and have therefore been consumed safely for countless generations. Their chemical structures tend to be complex, and can be used to classify them as shown in Table 9.2.

9.2.1 Heme Compounds

Heme pigments are responsible for the color of meat. Myoglobin (Mb) is the primary pigment and hemoglobin, the pigment of blood, is of secondary importance. Most of the hemoglobin is removed when animals are slaughtered and bled. Thus, in properly bled muscle tissue Mb is responsible for 90% or more of the pigmentation. The Mb quantity varies considerably among muscle tissues and is influenced by species, age, sex, and physical activity. For example, pale-colored veal has a lower Mb content than red-colored beef. Muscle-to-muscle differences within an animal also are apparent, and these differences are caused by varying quantities of Mb present within the muscle fibers. Such is the case with poultry, where light-colored breast muscle is easily distinguished from the dark muscle color of leg and thigh muscles. Listed in Table 9.3 are the major pigments found in fresh, cured, and cooked meat. Other minor pigments present in muscle tissue include the cytochrome enzymes, flavins, and vitamin B_{12}.

9.2.1.1 Myoglobin/Hemoglobin

9.2.1.1.1 Structure of Heme Compounds

Myoglobin is a globular protein consisting of a single polypeptide chain. Its molecular mass is 16.8 kD and it is comprised of 153 amino acids. This protein portion of the molecule is known as globin.

TABLE 9.2
Classification of Plant and Animal Pigments Based on Their Chemical Structure

Chemical Group	Pigment	Examples	Coloration	Occurrence (examples)
Tetrapyrroles	**Heme compounds**	Oxymyoglobin	Red	Fresh meats
		Myoglobin	Purple/red	
		Metmyoglobin	Brown	Packaged meats
	Chlorophylls	Chlorophyll *a*	Blue–green	Broccoli, lettuce, spinach
		Chlorophyll *b*	Green	
Tetraterpenoids	**Carotenoids**	Carotene	Yellow–orange	Carrots, oranges, peaches, peppers
		Lycopene	Orange–red	Tomatoes
O-heterocyclic compounds/quinones	**Flavonoids/phenolics**	Anthocyanins	Orange/red/blue	Berries, red apple, red cabbage, radish
		Flavonols	White–yellow	Onions, cauliflower
		Tannins	Red–brown	Aged Wine
N-heterocyclic compounds	**Betalains**	Betanin	Purple/red	Red Beets, swiss chard, cactus pear
		Betaxanthins	Yellow	

The chromophore component responsible for light absorption and color is a porphyrin known as heme. The porphyrin ring is formed by four pyrrole rings joined together and linked to a central iron atom. Thus, Mb is a complex of globin and heme (Figure 9.2). The heme porphyrin is present within a hydrophobic pocket of the globin protein and bound to a histidine residue (Figure 9.3). The centrally located iron atom shown possesses six coordination sites, four of which are occupied by the nitrogen atoms within the tetrapyrrole ring. The fifth coordination site is bound by the histidine residue of globin, leaving the sixth site available to complex with electronegative atoms donated by various ligands.

Hemoglobin consists of four Mbs linked together as a tetramer. Hemoglobin, a component of red blood cells, forms reversible complexes with oxygen in the lung. This complex is distributed via the blood to various tissues throughout the animal where oxygen is absorbed. It is the heme group that binds molecular oxygen. Mb within the cellular tissue acts in a similar fashion, accepting the oxygen carried by hemoglobin. Mb thus stores oxygen within the tissues, making it available for metabolism.

9.2.1.1.2 Chemistry and Color: Oxidation State

Meat color is determined by the chemistry of Mb, its state of oxidation, type of ligands bounds to heme, and state of the globin protein. The heme iron within the porphyrin ring may exist in two forms: either reduced ferrous (+2) or oxidized ferric (+3). This state of oxidation for the iron atom within heme should be distinguished from oxygenation of Mb. When molecular oxygen binds to Mb, oxymyoglobin (MbO_2) is formed and this is referred to as oxygenation. When oxidation of Mb occurs, the iron atom is converted to the ferric (+3) state, forming metmyoglobin (MMb). Heme iron in the +2 (ferrous) state that lacks a bound ligand in the sixth position is called myoglobin.

Meat tissue that contains primarily Mb (also referred to as deoxymyoglobin) is purplish-red in color. Binding of molecular oxygen at the sixth ligand yields MbO_2 and the color of the tissue changes to the customary bright red. Both the purple Mb and the red MbO_2 can oxidize, changing the state of the iron from ferrous to ferric. If this change in state occurs through autooxidation, these pigments acquire the undesirable brownish-red color of MMb. In this state, MMb is not capable of binding oxygen and the sixth coordination position is occupied by water [35]. Meat MMb can

TABLE 9.3
Major Pigments Found in Fresh, Cured, and Cooked Meat

Pigment	Mode of Formation	State of Iron	State of Hematin Nucleus	State of Globin	Color
1. Myoglobin	Reduction of metmyoglobin; deoxygenation of oxymyoglobin	Fe^{2+}	Intact	Native	Purplish-red
2. Oxymyoglobin	Oxygenation of myoglobin	Fe^{2+}	Intact	Native	Bright red
3. Metmyoglobin	Oxidation of myoglobin, oxymyoglobin	Fe^{3+}	Intact	Native	Brown
4. Nitric oxide myoglobin (nitrosomyoglobin)	Combination of myoglobin with nitric oxide	Fe^{2+}	Intact	Native	Bright red (pink)
5. Nitric oxide metmyoglobin (nitrosometmyoglobin)	Combination of metmyoglobin with nitric oxide	Fe^{3+}	Intact	Native	Crimson
6. Metmyoglobin nitrite	Combination of metmyoglobin with excess nitrite	Fe^{3+}	Intact	Native	Reddish-brown
7. Globin myohemochromogen	Effect of heat, denaturing agents on myoglobin, oxymyoglobin; irradiation of globin hemichromogen	Fe^{2+}	Intact (usually bound to denatured protein other than globin)	Denatured (usually detached)	Dull red
8. Globin myohemichromogen	Effect of heat, denaturing agents on myoglobin, oxymoglobin, metmyoglobin, hemochromogen	Fe^{3+}	Intact (usually bound to denatured protein other than globin)	Denatured (usually detached)	Brown (sometimes greyish)
9. Nitric oxide myohemochromogen	Effect of heat, denaturing agents on nitric oxide myoglobin	Fe^{2+}	Intact	Denatured	Bright red (pink)
10. Sulfmyoglobin	Effect of H_2S and oxygen on myoglobin	Fe^{3+}	Intact but one double bond saturated	Native	Green
11. Mtsulfmyoglobin	Oxidation of sulfmyoglobin	Fe^{3+}	Intact but one double bond saturated	Native	Red
12. Choleglobin	Effect of hydrogen peroxide on myoglobin or oxymyoglobin; effect of ascorbine or other reducing agent on oxymyoglobin	Fe^{2+} or Fe^{3+}	Intact but one double bond saturated	Native	Green
13. Nitrihemin	Effect of large excess nitrite and heat on 5	Fe^{3+}	Intact but reduced	Absent	Green
14. Verdohaem	Effect of reagents as in 7–9 in excess	Fe^{3+}	Porphyrin ring opened	Absent	Green
15. Bile pigments	Effect of reagents as in 7–9 in large excess	Fe absent	Porphyrin ring destroyed Chain of porphyrins	Absent	Yellow or colorless

Source: From Lawrie, R. A. (1985). *Meat Science*, 4th edn. Pergamon Press, New York.

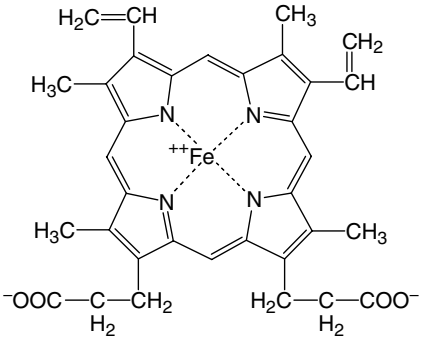


FIGURE 9.2 Chemical structure of heme.

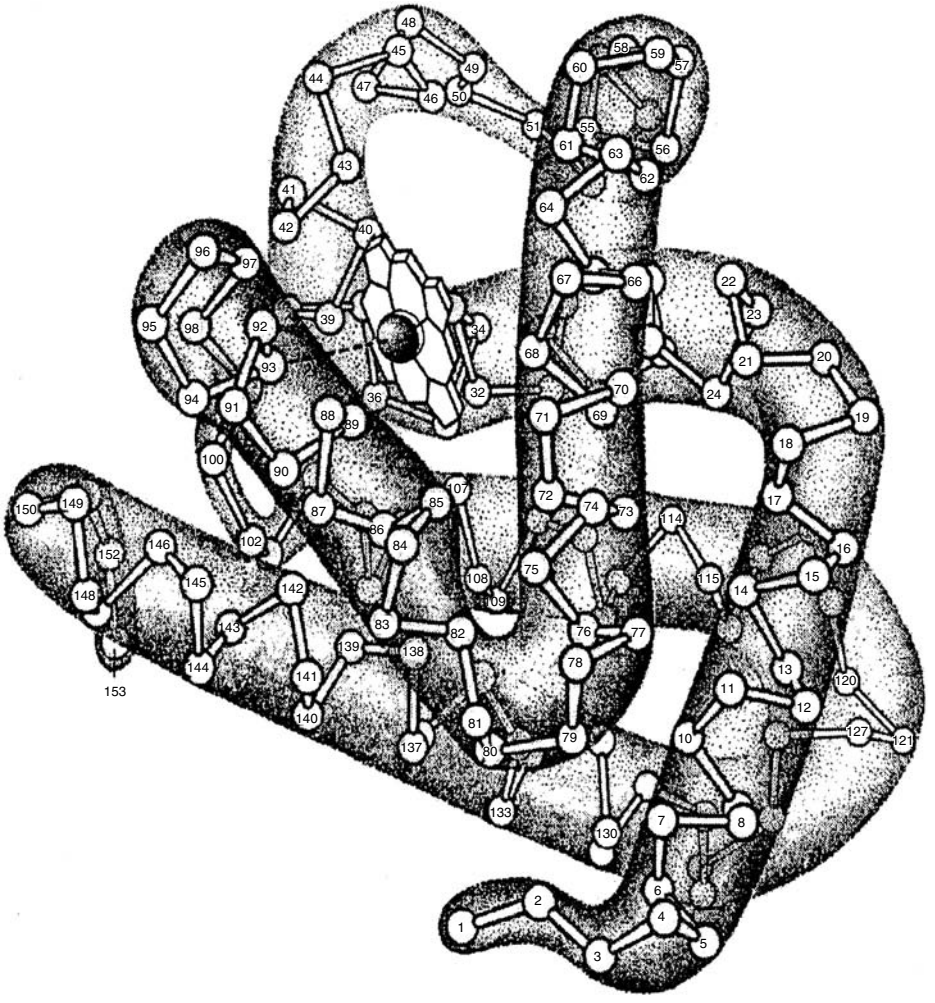


FIGURE 9.3 Tertiary structure of myoglobin. (Geis, I. (1983). *Biochemistry*, 2nd edn. F. B. Armstrong (Ed.). Oxford University Press, New York, p. 108.)

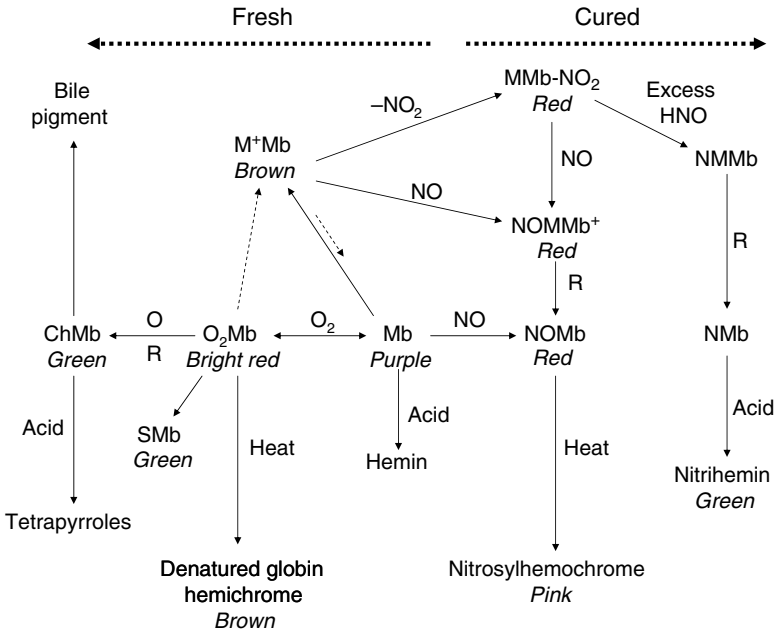


FIGURE 9.4 Myoglobin reactions in fresh and cured meats. ChMb = cholemyoglobin (oxidized porphyrin ring); O_2Mb = oxymyoglobin (Fe^{2+}); MMb = metmyoglobin (Fe^{3+}); Mb = myoglobin (Fe^{2+}); MMb-NO_2 = metmyoglobin nitrite; NOMMb = nitrosylmetmyoglobin; NOMb = nitrosylmyoglobin; NMMb = nitrometmyoglobin; NMb = nitromyoglobin, the latter two being reaction products of nitrous acid and the heme portion of the molecule; SMb = sulfomyoglobin; R = reductant; O = strong oxidizing conditions. (From Fox, J. B., Jr. (1966). *J. Agric. Food Chem.* 14:207–210.)

be reduced back to Mb both enzymatically and nonenzymatically. The main pathway seems to be by action of a MMb reductase that in the presence of NADH can effectively reduce MMb to the ferrous state [57,99,103]. Shown in Figure 9.4 are the various reactions of the heme pigment. Color reactions in fresh meat are dynamic and determined by conditions in the muscle and the resulting ratios of Mb, MMb, and MbO_2. While interconversion among Mb and MbO_2 can occur readily (and spontaneously) depending on oxygen tension, the conversion of MMb to the other forms would require enzymatic or nonenzymatic reduction of the ferric to the ferrous state.

Shown in Figure 9.5 is the relationship between oxygen partial pressure and the percentage of each type of heme pigment. A high partial pressure of oxygen favors oxygenation, forming bright-red MbO_2. Freshly cut meats will "bloom" or rapidly exhibit a bright-red coloration, a result of the rapid conversion of Mb to MbO_2 when exposed to oxygen in the environment. Conversely, at low oxygen partial pressures Mb and MMb are favored. In order to enhance MbO_2 formation, saturation levels of oxygen in the environment are useful. The rate of MMb formation, caused by heme oxidation (Fe^{2+} Fe^{3+}), can be minimized if oxygen is totally excluded. Muscles have varying oxygen partial pressures, causing the ratios of each of the pigment forms to vary.

Presence of the globin protein is known to decrease the rate of heme oxidation (Fe^{2+} Fe^{3+}). In addition, oxidation occurs more rapidly at lower pH values. Furthermore, the rate of autoxidation of MbO_2 occurs more slowly than that of Mb. The presence of trace metals, especially copper ions, is known to promote autoxidation.

9.2.1.1.3 Chemistry and Color: Discoloration

Two different reactions can cause green discoloration of Mb [84]. Hydrogen peroxide can react with either the ferrous or ferric site of heme, resulting in choleglobin, a green-colored pigment. Also,

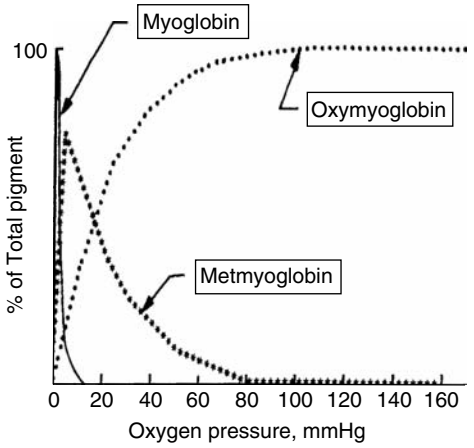


FIGURE 9.5 Influence of oxygen partial pressure on the three chemical states of myoglobin. (From Forrest, J. C., et al. (1975). *Principles of Meat Science*, W. H. Freeman, San Francisco.)

in the presence of hydrogen sulfide and oxygen, green sulfomyoglobin can form. It is thought that hydrogen peroxide and/or hydrogen sulfide arise from bacterial growth. A third mechanism for green pigmentation occurs in cured meats and is described in Section 9.2.1.2.

9.2.1.2 Cured Meat Pigments

In the manufacture of most cured meats, nitrates or nitrites are added to improve color and flavor and also to inhibit *Clostridium botulinum*. During the curing process, specific reactions occur that are responsible for the stable pink color of cured meat products. These reactions are outlined in Figure 9.4 and the compounds responsible for the reactions are listed in Table 9.3.

The first reaction occurs between nitric oxide (NO) and Mb to produce nitric oxide myoglobin (MbNO), also know as nitrosylmyoglobin. MbNO is bright red and unstable. Upon heating, the more stable nitric oxide myohemochromogen (nitrosylhemochrome) forms. This product yields the desirable pink color of cured meats. Heating of this pigment denatures globin, but the pink color persists. If MMb is present, it has been postulated that reducing agents are required to convert MMb to Mb before the reaction with NO can take place. Alternatively, nitrite can interact directly with MMb. In the presence of excess nitrous acid, nitrimyoglobin (NMb) will form. Upon heating in a reducing environment, NMb is converted to nitrihemin, a green pigment. This series of reactions causes a defect known as "nitrite burn."

In the absence of oxygen, NO complexes of Mb are relatively stable. However, especially under aerobic conditions, these pigments are sensitive to light. If reductants are added, such as ascorbate or sulfhydryl compounds, the reductive conversion of nitrite to NO is favored. Thus, under these conditions, formation of MbNO occurs more readily.

Parma Hams are a special type of ham manufactured using only pork and salt, without addition of nitrates or nitrites. During dry curing of these products a new pigment, Zn-protoporphyrin, is formed, in which the iron from heme is substituted by zinc [150]. These pigments are responsible for the stable bright red color of Parma Hams.

Detailed reviews on the chemistry of cured meat pigments are available [44,85,107].

9.2.1.3 Stability of Meat Pigments

The main factor determining consumer acceptance of meats is muscle color. Many factors operative in a complex food system can influence the stability of meat pigments. In addition, interactions

between these factors are critical and make it difficult to determine cause and effect relationships. Some environmental conditions that have important effects on meat color and pigment stability include exposure to light, temperature, relative humidity, pH, and the presence of specific bacteria. Review papers on this subject are available [35,80].

Specific reactions, such as lipid oxidation, are known to increase the rate of pigment oxidation [36]. Similarly, color stability can be improved by the addition of antioxidants such as ascorbic acid, vitamin E, butylated hydroxyanisole (BHA), or propyl gallate [54]. Vitamin E supplementation of beef cattle diets is an effective procedure for enhancing the lipid and color stability of meat products subsequently obtained from these animals [34]. These compounds have been shown to delay lipid oxidation and improve retention of color in tissues. Other biochemical factors, such as the rate of oxygen consumption prior to slaughter and activity of MMb reductase, can influence the color stability of fresh meat [92].

Irradiation of meats can also cause color changes because of the susceptibility of the Mb molecule, especially the iron, to alteration in the chemical environment and to energy input. Stable red pigments, brown pigment, and even green discoloration may occur during irradiation. A combination of preslaughter feeding of livestock with antioxidants, optimizing the conditions of the meat prior to irradiation, addition of antioxidants, use of modified atmosphere packaging (MAP), and controlling temperature may all contribute to optimize color during irradiation [12].

Many consumers use the internal cooked appearance of meat (i.e., ground beef patties) to evaluate doneness. However, two phenomena prevent using internal cooked color as an indicator: premature browning and persistent pink color. In premature browning, meat appears cooked (brown), even though it has not reached the internal temperature to kill pathogens. On the other hand, the color of some meats remains pink even after reaching safe internal cooking temperatures, so consumers overcook them. Therefore, it is important to understand that meat color should not be used as an indicator of meat doneness [64,76].

9.2.1.4 Packaging Considerations

An important means of stabilizing meat color is to store it under appropriate atmospheric conditions. The use of MAP can extend the shelf life of meat products. This technique requires the use of packaging films with low gas permeabilities. After packaging, air is evacuated from the package and the storage gas is injected, creating conditions that minimize the discoloration caused by heme oxidation ($Fe^{2+} \rightarrow Fe^{3+}$). By employing oxygen-enriched or -devoid atmospheres, color stability can be enhanced [101]. Muscle tissue stored under conditions devoid of O_2 (100% CO_2) and in the presence of an oxygen scavenger also exhibits good color stability [115,141]. Use of MAP techniques may, however, result in other chemical and biochemical alterations that can influence the acceptability of meat products. Part of the influence of modified atmospheres on pigment stability no doubt relates to its influence on microbial growth. Combinations of oxygen, carbon dioxide, and nitrogen have been used to maintain the quality of fresh red meat to optimize both microbiological and organoleptic quality. Addition of low levels of CO has resulted in extended shelf life though formation of carboxymyoglobin (MbCO) that is more stable to oxidation than MbO_2 and gives an attractive cherry-red color to meat [91]. Further information on use of MAP for fresh meat storage can be found in a review article by Seidman and Durland [127] and in Luño et al. [91].

9.2.2 Chlorophyll

9.2.2.1 Structure of Chlorophylls and Derivatives

Chlorophylls are the major light-harvesting pigments in green plants, algae, and photosynthetic bacteria. They are magnesium complexes derived from porphin. Porphin is a fully unsaturated

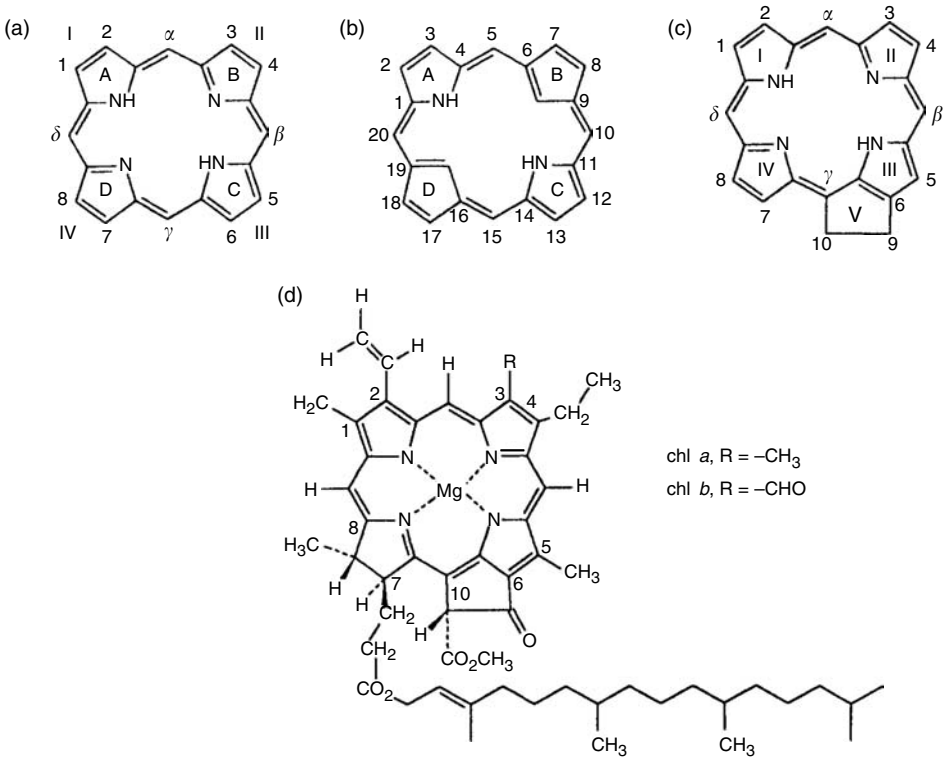


FIGURE 9.6 Structure of (a,b) porphin, (c) phorbin, and (d) chlorophyll (chl).

macrocyclic structure that contains four pyrrole rings linked by single bridging carbons. The rings are numbered I through IV or A through D according to the Fisher numbering system (Figure 9.6). Pyrrole carbon atoms on the periphery of the porphin structure are numbered 1 through 8. Carbon atoms of the bridging carbons are designated α, β, γ, and δ. The IUPAC numbering system for porphin is shown in Figure 9.6b. The more common numbering system is the Fisher system.

Substituted porphins are called porphyrins. A porphyrin is any macrocyclic tetrapyrrole pigment in which the pyrrole rings are joined by methyne bridges and the system of double bonds forms a closed, conjugated loop. Phorbin (Figure 9.6c) is considered to be the nucleus of all chlorophylls and is formed by the addition of a fifth isocyclic ring (V) to porphin. Chlorophylls, therefore, are classified as porphyrins.

Several chlorophylls are found in nature. Their structures differ in the substituents around the phorbin nucleus. Chlorophyll *a* and *b* are found in green plants in an approximate ratio of 3:1. They differ in the carbon C-3 substituent. Chlorophyll *a* contains a methyl group while chlorophyll *b* contains a formyl group (Figure 9.6d). Both chlorophylls have a vinyl and an ethyl group at the C-2 and C-4 position, respectively; a carbomethoxy group at the C-10 position of the isocylic rings, and a phytol group esterified to propionate at the C-7 position. Phytol is a 20-carbon monounsaturated isoprenoid alcohol. In Figure 9.6d, chlorophyll *c* is found in association with chlorophyll *a* in marine algae, dinoflagellates, and diatoms. Chlorophyll *d* is a minor constituent accompanying chlorophyll *a* in red algae. Bacteriochlorophylls and chlorobium chlorophylls are principal chlorophylls found in purple photosynthetic bacteria and green sulfur bacteria, respectively. Trivial names are widely used for chlorophylls and their derivatives [69]. Listed in Table 9.4 are the names used most often. Figure 9.7 is a schematic representation of the structural relationships of chlorophyll and some of its derivatives [121].

TABLE 9.4
Nomenclature of Chlorophyll Derivatives

Phyllins	Chlorophyll derivatives containing magnesium
Pheophytins	The magnesium-free derivatives of the chlorophylls
Chlorophylllides	The products containing a C-7 propionic acid resulting from enzymic or chemical hydrolysis of the phytyl ester
Pheophorbides	The products containing a C-7 propionic acid resulting from removal of magnesium and hydrolysis of the phytyl ester
Methyl or ethyl pheophorbides	The corresponding 7-propionate methyl of ethyl ester
Pyro compounds	Derivatives in which the C-10 carbomethoxy group has been replaced by hydrogen
Meso compounds	Derivatives in which the C-2 vinyl group has been reduced to ethyl
Chlorins e	Derivatives of pheophorbide *a* resulting from cleavage of the isocyclic ring
Rhodins g	The corresponding derivatives from pheophorbide *b*

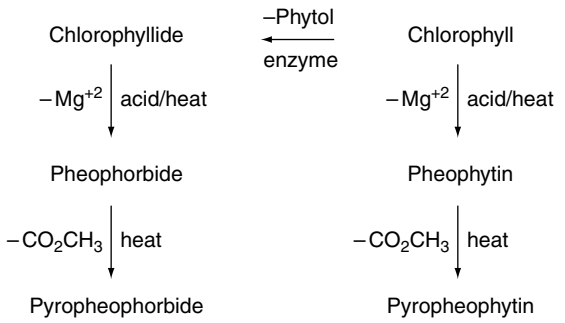


FIGURE 9.7 Chlorophyll and its derivatives.

9.2.2.2 Physical Characteristics

Chlorophylls are located in the lamellae of intercellular organelles of green plants known as chloroplasts. They are associated with carotenoids, lipids, and lipoproteins. Weak linkages (noncovalent bonds) exist between these molecules. The bonds are easily broken; hence chlorophylls can be extracted by macerating plant tissue in organic solvents. Polar solvents such as acetone, methanol, ethanol, ethyl acetate, pyridine, and dimethylformamide are most effective for complete extraction of chlorophylls. Nonpolar solvents such as hexane or petroleum ether are less effective. High-performance liquid chromatography (HPLC) today is the method of choice for separating individual chlorophylls and their derivatives. In the case of chlorophyll *a* and *b*, for example, the increase in polarity contributed by the C-3 formyl substituent of chlorophyll *b* causes it to be more strongly adsorbed on a normal-phase column and more weakly absorbed on a reverse-phase column than chlorophyll *a* [19,37,118].

Identification of chlorophyll and its derivatives is, to a large extent, based on absorption characteristics of visible light. Visible spectra of chlorophyll *a* and *b* and their derivatives are characterized by sharp light absorption bands between 600 and 700 nm (red regions) and between 400 and 500 nm (blue regions) (Table 9.5). The wavelength of maximum absorption for chlorophylls *a* and *b* dissolved in ethyl ether are, respectively, 660.5 and 642 nm in the red region and 428.5 and 452.5 nm in the blue region [132]. Recently, mass spectroscopic techniques such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) have been used for structure elucidation of chlorophyll allomers and their derivatives, produced during fruit and vegetable processing [65].

TABLE 9.5
Spectral Properties in Ethyl Ether of Chlorophyll *a* and *b* and Their Derivatives

Compound	Absorption Maxima (nm)		Ratio of Absorption ("Blue"/"Red")	Molar Absorptivity ("Red" Region)
	"Red" Region	"Blue" Region		
Chlorophyll *a*	660.5	428.5	1.30	86,300[a]
Methyl chlorophyllide *a*	660.5	427.5	1.30	83,000[b]
Chlorophyll *b*	642.0	452.5	2.84	56,100[a]
Methyl chlorophyllide *b*	641.5	451.0	2.84	—[b]
Pheophytin *a*	667.0	409.0	2.09	61,000[b]
Methyl pheophorbide *a*	667.0	408.5	2.07	59,000[b]
Pheophytin *b*	655	434	—	37,000[c]
Pyropheophytin *a*	667.0	409.0	2.09	49,000[b]
Zinc pheophytin *a*	653	423	1.38	90,300[d]
Zinc pheophytin *b*	634	446	2.94	60,200[d]
Copper pheophytin *a*	648	421	1.36	67,900[d]
Copper pheophytin *b*	627	438	2.53	49,800[d]

[a] Strain et al. [132].
[b] Pennington et al. [109].
[c] Davidson [27].
[d] Jones et al. [74].

9.2.2.3 Alterations of Chlorophyll

9.2.2.3.1 Enzymatic

Chlorophyllase is the only enzyme known to catalyze the degradation of chlorophyll. Chlorophyllase is an esterase, and *in vitro*, it catalyzes cleavage of phytol from chlorophylls and its Mg-free derivatives (pheophytins), forming chlorophyllides and pheophorbides, respectively (Figure 9.7). Its activity is limited to porphyrins with a carbomethoxy groups at C-10 and hydrogens at positions C-7 and C-8 [98]. The enzyme is active in solutions containing water, alcohols, or acetone. In the presence of large amounts of alcohols such as methanol or ethanol, the phytol group is removed and the chlorophyllide is esterified to form either methyl or ethyl chlorophyllide. Formation of chlorophyllides in fresh leaves does not occur until the enzyme has been heat activated postharvest. The optimum temperature for chlorophyllase activity in vegetables ranges between 60 and 82.2°C [88]. Enzyme activity decreases when plant tissue is heated above 80°C, and chlorophyllase loses its activity if heated to 100°C. Chlorophyllase activity in spinach during growth and fresh storage is shown in Figure 9.8. Maximum activity is observed at the time the plant begins flowering (broken line). Postharvest storage of fresh spinach at 5°C decreases enzyme activity compared to activities measured at the time of harvest (solid line) [122].

9.2.2.3.2 Heat and Acid

Chlorophyll derivatives formed during heating or thermal processing can be classified into two groups based on the presence or absence of the magnesium atom in the tetrapyrrole center. Mg-containing derivatives are green in color, while Mg-free derivatives are olive-brown in color. The latter are chelators and when, for example, sufficient zinc or copper atoms are available they will form green zinc or copper complexes.

The first change observed when the chlorophyll molecule is exposed to heat is isomerization. Chlorophyll isomers are formed by inversion of the C-10 carbomethoxy group. The isomers are

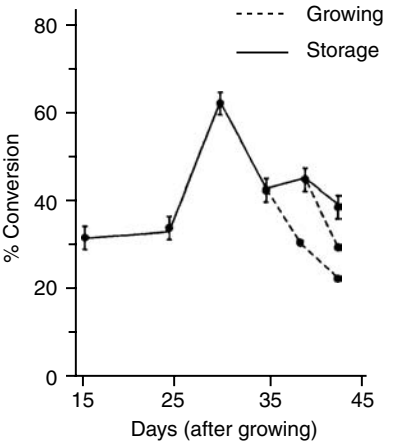


FIGURE 9.8 Chlorophyllase activity, expressed as percent conversion of chlorophyll to chlorophyllide, in spinach during growth (broken line) and after storage at 5°C (solid line). (From von Elbe, J. H. and L. F. LaBorde (1989). In *Quality Factors of Fruit and Vegetables Chemistry and Technology*. J. J. Jen (Ed.). Am. Chem. Soc. Symp. Ser. 405, Washington, D.C., pp. 12–28.)

designated as a' and b'. They are more strongly absorbed on a C-18 reverse-phase HPLC column than are their parent compounds, and clear separation can be achieved. Isomerization occurs rapidly in heated plant tissue or in organic solvents. Establishment of equilibrium in leaves results in conversion of 5–10% of chlorophyll *a* and *b* to a' and b' after heating for 10 min at 100°C [5,122,151]. Chromatograms of a spinach extract of fresh vs. blanched spinach in Figure 9.9 show isomer formation during heating.

The magnesium atom in chlorophyll is easily displaced by two hydrogen atoms, resulting in the formation of olive-brown pheophytin (Figure 9.10). The reaction is irreversible in aqueous solution. Compared to their parent compounds, pheophytin *a* and *b* are less polar and more strongly absorbed on a reverse-phase HPLC column. Formation of the respective pheophytins occur more rapidly from chlorophyll *a* than from chlorophyll *b*. Chlorophyll *b* is more heat stable than chlorophyll *a*. The greater stability of chlorophyll *b* is attributed to the electron-withdrawing effect of its C-3 formyl group. Transfer of electrons away from the center of the molecule occurs because of the conjugated structure of chlorophyll. The resulting increase in positive charge on the four pyrrole nitrogens reduces the equilibrium constant for the hydrogenation of the pyrrole nitrogen. Reported activation energies for the reaction range from 12.6 to 35.2 kcal/mol. This variance has been attributed to differences in media composition, pH, and temperature range.

Chlorophyll degradation in heated vegetable tissue is affected by tissue pH. In a basic media (pH 9.0) chlorophyll is very stable toward heat, whereas in an acidic media (pH 3.0) it is unstable. A decrease of 1 pH unit can occur during heating of plant tissue through the release of acids, and this has an important detrimental effect on the rate of chlorophyll degradation. Pheophytin formation in intact plant tissue, postharvest, appears to be mediated by cell membrane disrupton. In a study by Haisman and Clarke [58], chlorophyll degradation in sugar beet leaves held in heated buffer was not initiated until the temperature reached 60°C or higher. Conversion of chlorophyll to pheophytin after holding for 60 min at 60 or 100°C was 32 and 97%, respectively. It was proposed that pheophytin formation in plant cells is initiated by a heat-induced increase in permeability of hydrogen ions across cell membranes. The critical temperature for initiation of pheophytin formation coincided with gross changes in membrane organization as observed with an electron microscope.

The addition of chloride salts of sodium, magnesium, or calcium decreases pheophytin formation in tobacco leaves heated at 90°C by about 47, 70, and 77%, respectively. The decrease in chlorophyll degradation was attributed to the electrostatic shielding effect of the salts. It was proposed that the

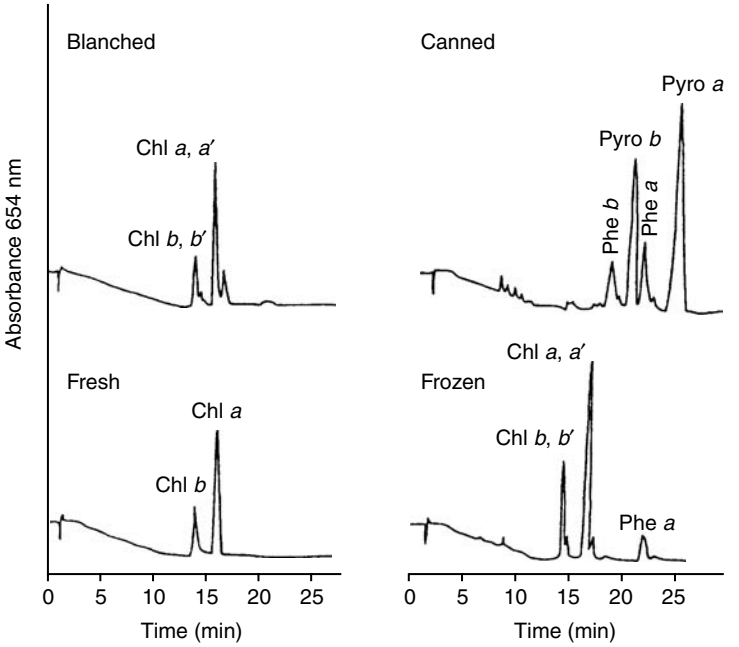


FIGURE 9.9 High-performance liquid chromatography (HPLC) chromatograms of chlorophylls (chl) and derivatives in fresh, blanched, frozen, and canned spinach. Phe = pheophytin, Pyro = pyropheophytin. (From Schwartz, S. J., et al. (1981). *J. Agric. Food Chem.* 29:533–535.)

addition of cations neutralizes the negative surface charge of the fatty acids and protein in the chloroplast membrane and thereby reduces the attraction of hydrogen ions to the membrane surface [100].

The permeability of hydrogen across the membrane can also be affected by the addition of detergents that adsorb on the surface of the membrane. Cationic detergents repel hydrogen ions at the membrane surface, limiting their diffusion into the cell and thereby decreasing chlorophyll degradation. Anionic detergents attract hydrogen ions, increasing their concentration at the membrane surface, increasing the rate of hydrogen diffusion, and increasing the degradation of chlorophyll. In the case of neutral detergents, the negative surface charge on the membrane is diluted and therefore the attraction for hydrogen ions and consequent degradation of chlorophyll is decreased [22,58].

Replacement of the C-10 carbomethoxy group of pheophytin with a hydrogen atom results in the formation of olive-colored pyropheophytin. The wavelengths of maximum light absorption by pyropheophytin are identical to those for pheophytin in both the red and blue regions (Table 9.5). Retention times for pyropheophytin *a* and *b* using reverse-phase HPLC are greater than those for the respective pheophytins.

Chlorophyll alteration during heating is sequential, and proceeds according to the following kinetic sequence:

$$\text{Chlorophyll} \rightarrow \text{Pheophytin} \rightarrow \text{Pyropheophytin}.$$

The data in Table 9.6 show that during heating for the first 15 min, chlorophyll decreases rapidly and pheophytin increases rapidly. With further heating pheophytin decreases and pyropheophytin rapidly increases, although a small amount of pyropheophytin is evident after 4 min of heating. Accumulation does not become appreciable until after 15 min, thus supporting a sequential mechanism. First-order rate constant for conversion of pheophytin *b* to pyropheophytin *b* is 25–40% greater than that for conversion of pheophytin *a* to pyropheophytin *a* [91]. Activation energies for removal of the C-10 carbomethoxy group from either pheophytin *a* and *b* are smaller than those for formation of

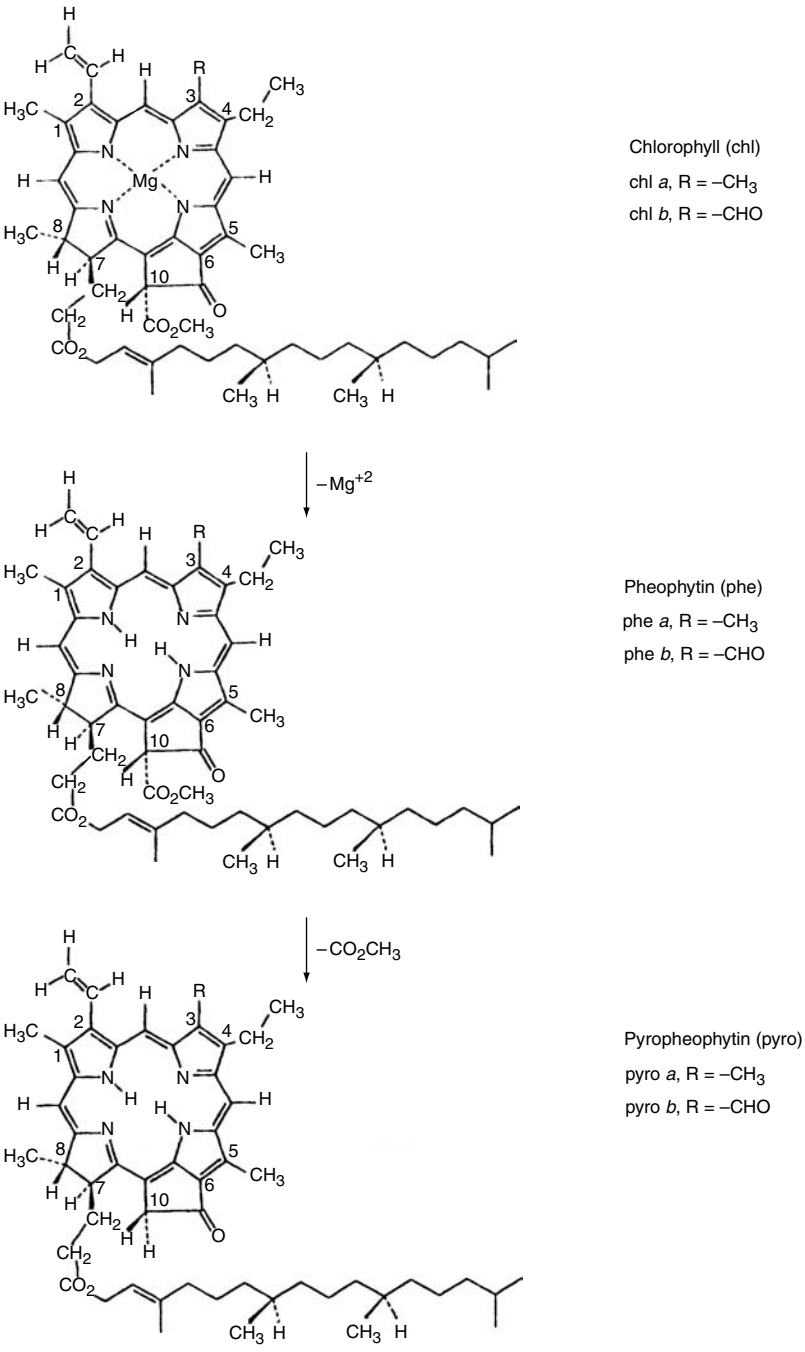


FIGURE 9.10 Formation of pheophytin and pyropheophytin from chlorophyll.

pheophytin *a* and *b*, from chlorophyll *a* and *b*, indicating a slightly lower temperature dependency for formation of the pyropheophytins from pheophytins.

Listed in Table 9.7 are the concentrations of pheophytins *a* and *b* and pyropheophytins *a* and *b* in commercially canned vegetable products. These data indicate that pyropheophytins *a* and *b* are the major chlorophyll derivatives responsible for the olive-green color in many canned vegetables. It is also significant that the amount of pyropheophytin derivatives formed is an indication of the severity

TABLE 9.6
Concentration (mg/g Dry Weight)[a] of Chlorophylls, Pheophytins and Pyropheophytins *a* and *b* in Fresh, Blanched, and Heated Spinach Processed at 121°C for Various Times

	Chlorophyll		Pheophytin		Pyropheophytin		
	a	***b***	***a***	***b***	***a***	***b***	**pH[b]**
Fresh	6.98	2.49					
Blanched	6.78	2.47				7.06	
Processed (min)[c]							
2	5.72	2.46	1.36	0.13			6.90
4	4.59	2.21	2.20	0.29	0.12		6.77
7	2.81	1.75	3.12	0.57	0.35		6.60
15	0.59	0.89	3.32	0.78	1.09	0.27	6.32
30		0.24	2.45	0.66	1.74	0.57	6.00
60			1.01	0.32	3.62	1.24	5.65

[a] Estimated error ±2%; each value represents a mean of 3 determinations.
[b] The pH was measured after processing and before pigment extraction.
[c] Times listed were measured after the internal product temperature reached 121°C.

Source: Luh, B. S., et al. (1964). *Food Technol.* 18:363–366.

TABLE 9.7
Pheophytins and Pyropheophytins *a* and *b* in Commercially Canned Vegetables

	Pheophytin[a] (μg/g dry weight)		Pyropheophytin (μg/g dry weight)	
Product	***a***	***b***	***a***	***b***
Spinach	830	200	4000	1400
Beans	340	120	260	95
Asparagus	180	51	110	30
Peas	34	13	33	12

[a] Estimated error ±2%.

Source: Luh, B. S., et al. (1964). *Food Technol.* 18:363–366.

of the heat treatment. Comparative heat treatments for commercial sterility of spinach, green beans, cut asparagus, and green peas in 303 cans processed at 121°C are approximately 51, 11, 13, and 17 min, respectively. From Table 9.7, the percents of pyropheophytin compared to the total pheo compounds are 84, 44, 38, and 49%, respectively, corresponding fairly well to the heating times.

Replacement of the magnesium atom in chlorophyllide (green) with hydrogen ions results in the formation of olive-brown pheophorbide. Pheophorbide *a* and *b* are more water soluble than the respective pheophytins and have the same spectral characteristics. Removal of the C-10 phytol chain appears to affect the rate of loss of magnesium from the tetrapyrrole center. Degradation rate of chlorophyllides *a* and *b* and their respective methyl and ethyl esters in acidic acetone increases as the length of the chain is decreased, suggesting that steric hindrance from the C-10 chain affects the rate of hydrogen ion attack [116].

Past rate studies using green plant tissue have generally supported the view that chlorophyllides are slightly more heat stable than chlorophylls. More recently [20], however, it was shown that the

reverse is true. Chlorophyllide *a* degraded 3.7 times faster than chlorophyll *a* at 145°C. The more water-soluble chlorophyllide apparently is more likely to contact hydrogen ions in solution and is likely to react more rapidly. The latter result is believed to be the correct one because, in this study, chlorophyllase was activated more completely, and therefore greater amounts of chlorophyllides were available for kinetic studies, and a very sensitive HPLC technique was used.

9.2.2.3.3 Metallo Complex Formation

The two hydrogen atoms within the tetrapyrrole nucleus of the magnesium-free chlorophyll derivative are easily displaced by zinc or copper ions to form green metallo complexes. Formation of metallo complexes from pheophytins *a* and *b* causes the red maximum to shift to a shorter wavelength and the blue maximum to a longer wavelength (Table 9.5) [74]. Spectral characteristics of the phytol-free metal complexes are identical to their parent compounds.

The Zn and Cu complexes are more stable in acid than in alkaline solutions. Magnesium, as has been pointed out, is easily removed by the addition of acid at room temperature, while zinc pheophytin *a* is stable in solution at pH 2. Removal of copper is achieved only at pH values sufficiently low to begin degradation of the porphyrin ring.

Incorporation of metal ions into the neutral porphyrin is a bimolecular reaction. It is believed that the reaction begins with attachment of the metal ion to a pyrrole nitrogen atom, followed by the immediate and simultaneous removal of two hydrogen atoms. Formation of metallo complexes is affected by substituent groups because of the highly resonant structure of the tetrapyrrole nucleus [33].

Metallo complexes of chlorophyll derivatives are known to form in plant tissue, and the a complexes form faster than the b complexes. The slower formation of the b complexes has been attributed to the electron-withdrawing effect of the C-3 formyl group. Migration of electrons away from the conjugated porphyrin ring system causes pyrrole nitrogen atoms to become more positively charged and therefore less reactive with metal cations. Steric hindrance of the phytol chain also decreases the rate of complex formation. Pheophorbide *a* in ethanol reacts four times faster with copper ions than does pheophytin *a* [73].

The kinetics of zinc complex formation have been studied. Formation of Zn^{2+} pyropheophorbide *a* occurs most rapidly in acetone/water (80/20), followed by pheophorbide *a*, methyl pheophorbide *a*, ethyl pheophorbide *a*, pyropheophytin *a*, and pheophytin *a*. Reaction rates decrease as the length of the alkyl chain esterified at the C-7 carbon increases, suggesting that steric hindrance is important. Similarly, the greater rate of formation of Zn-pyropheophytin *a* as compared to pheophytin *a* is attributed to interference from the C-10 carbomethoxy group of pheophytin *a* [109,142].

Comparative studies on the formation of metallo complexes in vegetable purées indicate copper is chelated more rapidly than zinc. Copper complexes are detectable in pea purée when the concentration of copper is as low as 1–2 ppm. In contrast, zinc complex formation under similar conditions does not occur in purée containing less than 25 ppm Zn^{2+}. When both Zn^{2+} and Cu^{2+} are present, formation of copper complexes dominates [117].

The pH is also a factor in the rate of complex formation. Increasing the pH of spinach purée from 4.0 to 8.5 results in an 11-fold increase in the amount of zinc pyropheophytin *a* formed during heating for 60 min at 121°C. A decrease in the rate of complex formation occurs when the pH is raised to 10, presumably because of precipitation of Zn^{2+} [81].

These metallo complexes are of interest because the copper complexes, based on their stability under most conditions experienced in food processing, are used as colorants in the EEC. This technology is not yet approved in the United States. A process that improves the green color of canned vegetables is based on the formation of zinc metallo complexes. Green vegetables canned with this process were introduced into the United States in 1990.

9.2.2.3.4 Allomerization

Chlorophylls oxidize when dissolved in alcohol or other solvents and exposed to air. This process is referred to as allomerization. It is associated with uptake of oxygen equimolar to the chlorophylls

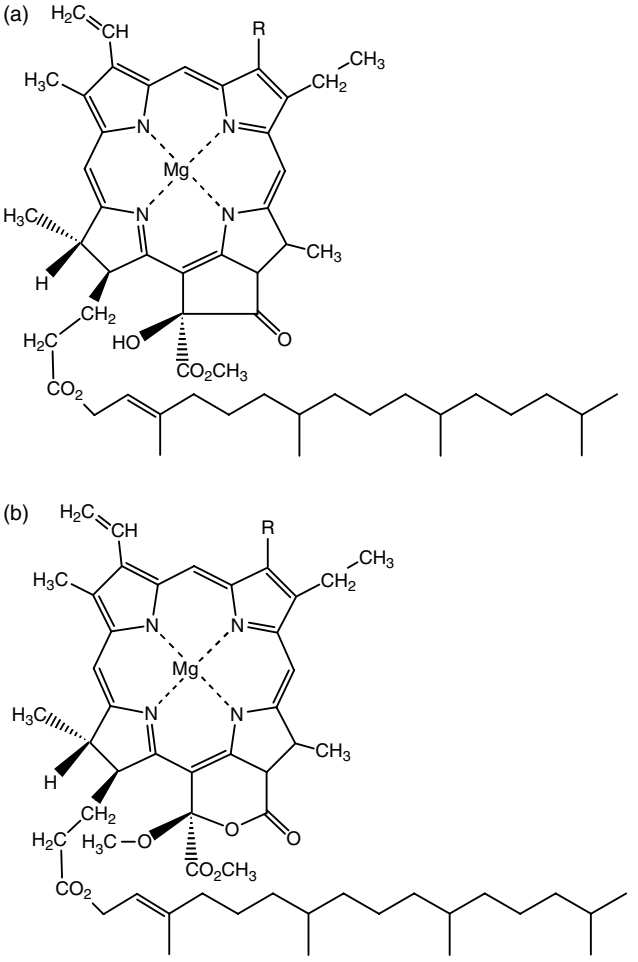


FIGURE 9.11 Structure of (a) 10-hydroxychlorophyll and (b) 10-methoxylactone of chlorophyll.

present [125]. The products are blue-green in color and do not give a yellow-brown ring in the Molisch phase test [60]. The lack of a color response indicates that the cyclopentanone ring (ring V, Figure 9.6c) has been oxidized or that the carbomethoxy group at C-10 has been removed. The products of allomerization have been identified as 10-hydroxychlorophylls and 10-methoxylactones (Figure 9.11). The principal allomerization product of chlorophyll *b* is the 10-methoxylactone derivative.

9.2.2.3.5 Photodegradation

Chlorophyll is protected from destruction by light during photosynthesis in healthy plant cells by surrounding carotenoids and other lipids. Chlorophyll can act as a sensitizer and generate singlet oxygen while carotenoids are known to quench this reactive oxygen species and protect the plant from photodegradation. Once this protection is lost by plant senescence, by pigment extraction from the tissue, or by cell damage caused during processing, chlorophylls are susceptible to photodegradation [86,87]. When these conditions prevail and light and oxygen are present, chlorophylls are irreversibly bleached.

Many researchers have tried to identify colorless photodegradation products of chlorophylls. Methyl ethyl maleimide has been identified by Jen and Mackinney [71,72]. In a study by Llewellyn

et al. [86,87] glycerol was found to be the major breakdown product, with lactic, citric, succininc, and malonic acids, and alanine occurring in lesser amounts. The reacted pigments were completely bleached.

It is believed that photodegradation of chlorophylls results in opening of the tetrapyrrole ring and fragmentation into lower molecular weight compounds. It has been suggested that photodegradation begins with ring opening at one of the methyne bridges to form oxidized linear tetrapyrroles [134]. Singlet oxygen and hydroxyl radicals are known to be produced during exposure of chlorophylls or similar porphyrins to light in the presence of oxygen [39]. Once formed, singlet oxygen or hydroxyl radicals will react further with tetrapyrroles to form peroxides and more free radicals, eventually leading to destruction of the porphyrins and total loss of color.

9.2.2.4 Color Loss During Thermal Processing

Loss of green color in thermally processed vegetables results from formation of pheophytin and pyropheophytin. Blanching and commercial heat sterilization can reduce chlorophyll content by as much as 80–100% [122]. Evidence that a small amount of pheophytin is formed during blanching before commercial sterilization is provided in Figure 9.9. The greater amount of pheophytin detected in frozen spinach as compared to spinach blanched for canning is most likely attributable to the greater severity of the blanch treatment that is generally applied to vegetables intended for freezing. One of the major reasons for blanching of spinach prior to canning is to wilt the tissue and facilitate packaging, whereas blanching prior to freezing must be sufficient not only to wilt the tissue but also to inactivate enzymes. The pigment composition shown for the canned sample indicated that total conversion of chlorophylls to pheophytins and pyropheophytins has occurred.

Degradation of chlorophyll within plant tissues postharvest is initiated by heat-induced decompartmentalization of cellular acids as well as the synthesis of new acids [58]. In vegetables several acids have been identified, including oxalic, malic, citric, acetic, succinic, and pyrrolidone carboxylic acid (PCA). Thermal degradation of glutamine to form PCA is believed to be the major cause of the increase in acidity of vegetables during heating [23]. Other contributors to increased acidity may be fatty acids formed by lipid hydrolysis, hydrogen sulfide liberated from proteins or amino acids, and carbon dioxide from browning reactions. The pH decrease occurring during thermal processing of spinach purée is shown in Table 9.6.

9.2.2.5 Technology of Color Preservation

Efforts to preserve green color in canned vegetables have concentrated on retaining chlorophyll, forming or retaining green derivatives of chlorophyll, that is, chlorophyllides, or creating a more acceptable green color through the formation of metallo complexes.

9.2.2.5.1 Acid Neutralization to Retain Chlorophyll

The addition of alkalizing agents to canned green vegetables can result in improved retention of chlorophylls during processing. Techniques have involved the addition of calcium oxide and sodium dihydrogen phosphate in blanch water to maintain product pH or to raise the pH to 7.0. Magnesium carbonate or sodium carbonate in combination with sodium phosphate has been tested for this purpose. However, all of these treatments result in softening of the tissue and an "alkaline" flavor.

Blair [10] in 1940 recognized the toughening effect of calcium and magnesium when added to vegetables. This observation led to the use of calcium or magnesium hydroxide for the purpose of raising pH and maintaining texture. This combination of treatments became known as the "Blair process." Commercial application of these processes has not been successful because of the inability of the alkalizing agents to effectively neutralize interior tissue acids over a long period of time, resulting in substantial color loss after less than 2 months of storage.

Another technique involved coating the can interior with ethylcellulose and 5% magnesium hydroxide. It was claimed that slow leaching of magnesium oxide from the lining would maintain the pH at or near 8.0 for a longer time and would therefore help stabilize the green color [93,94]. These efforts were only partially successful, because increasing the pH of canned vegetables can also cause hydrolysis of amides such as glutamine or asparagine with formation of undesirable ammonia-like odors. In addition, fatty acids formed by lipid hydrolysis during high-pH blanching may oxidize to form rancid flavors. In peas an elevated pH (8.0 or above) can cause formation of struvite, a glass-like crystals consisting of a magnesium and ammonium phosphate complex. Struvite is believed to result from the reaction of magnesium with ammonium generated from the protein in peas during heating.

9.2.2.5.2 High-Temperature Short-Time Processing

Commercially sterilized foods processed at a higher temperature than normal for a relatively short time (HTST) often exhibit better retention of vitamins, flavor, and color than do conventionally processed foods. The greater retention of these constituents in HTST foods results because their destruction is more temperature-dependent than that for inactivation of *C. botulinum* spores. Temperature dependence can be expressed in terms of z-value or activation energy. The z-value is the change in °C required to effect a tenfold change in the destruction rate. The z-values for the formation of pheophytins *a* and *b* in heated spinach purée have been determined to be 51 and 98°C, respectively [56]. The large values for both as compared to that for *C. botulinum* spores ($z = 10$°C), result in greater color retention when HTST processing is used [120]. However, this advantage of HTST processing is lost after about 2 months of storage, apparently because of a decrease in product pH during storage [90,138].

Other studies of vegetable tissue have combined HTST processing with pH adjustment. Samples treated in this manner were initially greener and contained more chlorophyll than control samples (typical processing and pH). However, the improvement in color, as previously mentioned, was generally lost during storage [56,16].

9.2.2.5.3 Enzymatic Conversion of Chlorophyll to Chlorophyllides to Retain Green Color

Blanching at lower temperatures than conventionally used to inactivate enzymes has been suggested as a means of achieving better retention of color in green vegetables, in the belief that the chlorophyllides produced have greater thermal stability than their parent compounds. Early studies showed that when spinach was blanched for canning at 71°C (160°F) for a total of 20 min better color retention resulted. This occurred as long as the blanch temperature was maintained between 54°C (130°F) and 76°C (168°F). It was concluded that the better color of processed spinach blanched under low temperature conditions (65°C for up to 45 min) was caused by heat-induced conversion of chlorophyll to chlorophyllides by the enzyme chlorophyllase [88]. However, the improvement in color retention achieved by this approach was insufficient to warrant commercialization of the process [22].

The progress of conversion of chlorophyll to chlorophyllides in heated spinach leaves is shown in Figure 9.12. The extract of unblanched spinach contained only chlorophylls *a* and *b*. Activation of chlorophyllase in spinach blanched at 71°C is illustrated by the formation of chlorophyllides (Figure 9.12b), while the absence of almost all chlorophyllides in spinach blanched at 88°C results from inactivation of the enzyme. These data also illustrate the previously mentioned point that isomerization of chlorophyll occurs during heating, as illustrated by the presence of a new peak corresponding to chlorophyll *a* isomer (Figure 9.12b and 9.12c) [148].

9.2.2.5.4 Commercial Application of Metallo Complex

Current efforts to improve the color of green processed vegetables and to prepare chlorophylls that might be used as food colorants have involved the use of either zinc or copper complexes of

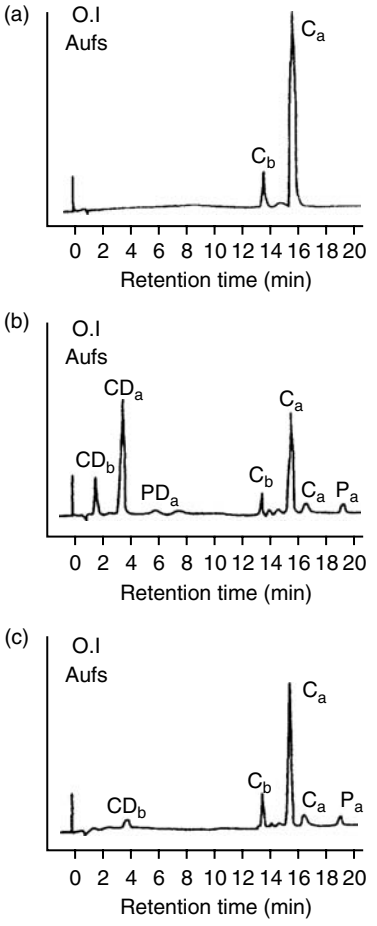


FIGURE 9.12 Chlorophyll and chlorophyll derivatives of spinach: (a) unblanched, (b) blanched 3 min at 71°C, and (c) blanched 3 min at 88°C. C_a = chlorophyll *a* (different retention times correspond to isomeric forms), C_b = chlorophyll *b*, P_a = pheophytin *a*, CD_a = chlorophyllide *a*, CD_b = chlorophyllide *b*, PD_a = pheophorbide *a*. (From von Elbe, J. H. and L. F. LaBorde (1989). In *Quality Factors of Fruit and Vegetables Chemistry and Technology*. J. J. Jen (Ed.). Am. Chem. Soc. Symp. Ser. 405, Washington, D.C., pp. 12–28.)

chlorophyll derivatives. Copper complexes of pheophytin and pheophorbide are available commercially under the names copper chlorophyll and copper chlorophyllin, respectively. These chlorophyll derivatives cannot be used in foods in the United States. Their use in canned foods, soups, candy, and dairy products is permitted in most European countries under regulatory control of the EEC. The Food and Agriculture Organization (FAO) of the United Nations has certified their use as safe in foods, provided no more than 200 ppm of free ionizable copper is present.

Commercial production of the Cu-chlorophylls was described by Humphry [63]. Chlorophyll is extracted from dried grass or alfalfa with acetone or chlorinated hydrocarbons. Sufficient water is added, depending on the moisture content of the plant material, to aid penetration of the solvent while avoiding activation of chlorophyllase. Some pheophytin forms spontaneously during extraction. Copper acetate is added to form oil-soluble copper chlorophyll. Alternatively, pheophytin can be acid hydrolyzed before copper ion is added, resulting in formation of water-soluble copper chlorophyllin (pyropheophytin). The copper complexes have greater stability than comparable Mg complexes; for example, after 25 h at 25°C, 97% of the chlorophyll degrades, while only 44% of the copper chlorophyll degrades.

9.2.2.5.5 Regreening of Thermal Processed Vegetables

It has been observed that when vegetables purées are commercially sterilized, small bright-green areas occasionally appear. It was determined that pigments in the bright-green areas contained zinc and copper. This formation of bright-green areas in vegetable purées was termed "regreening." Regreening of commercially processed vegetables has been observed when zinc and/or copper ions are present in process solutions. When okra is processed in brine solution containing zinc chloride, it retains its bright green color, and this is attributed to the formation of zinc complexes of chlorophyll derivatives [38,135,137].

A patent was issued to Continental Can Company (now Crown Cork & Seal Company) for the commercial canning of vegetables with metal salts in blanch or brine solution. The process involved blanching vegetables in water containing sufficient amounts of Zn^{2+} or Cu^{2+} salts to raise the tissue concentration of the metal ions to between 100 and 200 ppm. The direct addition of zinc chloride to the canning brine has no significant effect on the color of vegetables (green beans and peas). Green vegetables processed in modified blanch water were claimed to be greener than conventionally processed vegetables. Other bi- or trivalent metal ions were either less effective or ineffective [126]. This approach is known as the Veri-Green process. Pigments present in canned green beans processed by the Veri-Green process were identified as zinc pheophytin and zinc pyropheophytin [147].

Commercial production of zinc-processed green beans and spinach is presently conducted by several processors, but results with various vegetables have been mixed. Shown in Figure 9.13 is the sequence of pigment changes occurring when pea purée is heated in the presence of 300 ppm Zn^{2+}. Chlorophyll *a* decreases to trace levels after only 20 min of heating. Accompanying this rapid decrease in chlorophyll is the formation of zinc complexes of pheophytin *a* and pyropheophytin *a*. Further heating increases the zinc pyropheophytin concentration at the expense of a decrease in zinc pheophytin. In addition, zinc pyropheophytin may form through decarboxymethylation of zinc pheophytin or by reaction of pyropheophytin with Zn^{2+}. These results suggest that the green color in vegetables processed in the presence of zinc is largely due to the presence of zinc pyropheophytin.

Shown in Figure 9.14 is a proposed sequence of reactions for conversion of chlorophyll *a* to zinc pyropheophytin *a*. Formation of Zn-complexes occurs most rapidly between pH 4.0 and 6.0, and the rate decreases markedly at pH 8.0. The reason for the decrease is that chlorophyll is retained at the high pH, thereby limiting the amount of chlorophyll derivatives available for complex formation

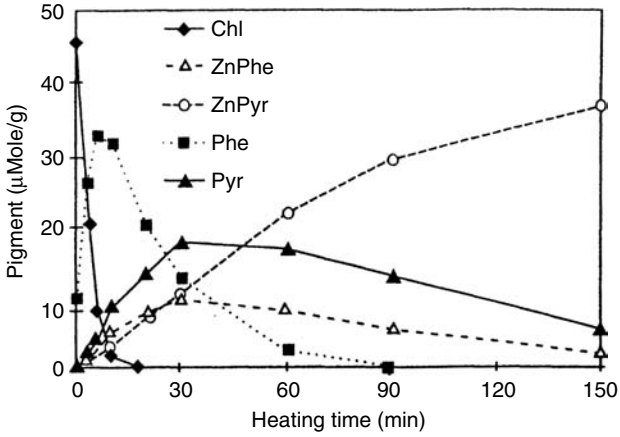


FIGURE 9.13 Pigments in pea purée containing 300 ppm of Zn^{2+} after heating at 121°C for up to 150 min. Chl = chlorophyll, ZnPhe = zinc pheophytin, ZnPyr = zinc pyropheophytin, Phe = pheophytin, Pyr = pyropheophytin. (From von Elbe, J. H. and L. F. LaBorde (1989). In *Quality Factors of Fruit and Vegetables Chemistry and Technology*. J. J. Jen (Ed.). Am. Chem. Soc. Symp. Ser. 405, Washington, D.C., pp. 12–28.)

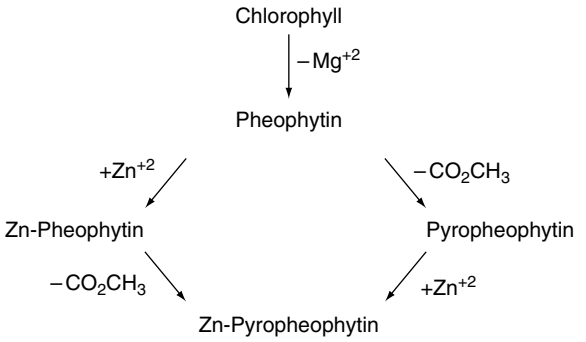


FIGURE 9.14 Chemical reactions occurring in heated green vegetables containing zinc.

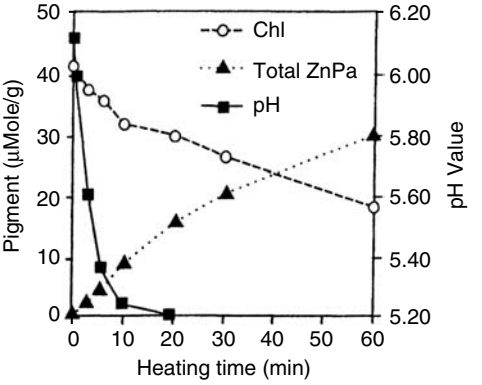


FIGURE 9.15 Conversion of chlorophyll *a* (chl) to total zinc complexes (ZnPa) and change in pH in pea purée heated at 121°C for up to 60 min. (From Haisman, D. R. and M. W. Clarke (1975). *J. Sci. Food Agric.* 26:1111–1126.)

(Figure 9.15) [82,83]. It has further been shown that zinc complex formation can be influenced by the presence of surface-active anionic compounds. Such compounds adsorb onto the chloroplast membranes, increasing the negative surface charge and thereby increasing complex formation.

Currently, the best process for attaining a desirable green color in canned vegetables involves adding zinc to the blanch solution, increasing membrane permeability by heating the tissue prior to blanching at or slightly above 60°C, choosing a pH that favors formation of metallo complexes, and using anions to alter surface charge on the tissue.

9.2.3 Carotenoids

Carotenoids are nature's most widespread pigments, with the earth's annual biomass production estimated at 100 million tons. A large majority of these pigments are biosynthesized by the ocean algae population. In higher plants, carotenoids in chloroplasts are often masked by the more dominant chlorophyll pigments. In the autumn season when chloroplasts decompose during plant senescence, the yellow-orange color of carotenoids becomes evident [7].

For several decades, it has been known that carotenoids play important functions in photosynthesis and photoprotection in plant tissues [53]. In all chlorophyll-containing tissues, carotenoids function as secondary pigments in harvesting light energy. The photoprotection role of carotenoids stems from their ability to quench and inactivate reactive oxygen species (particularly singlet oxygen) formed by exposure to light and air. In addition, specific carotenoids present in roots and leaves

serve as precursors to abscisic acid, a compound that functions as a chemical messenger and growth regulator [28,105].

The most prominent role of carotenoid pigments in the diet of humans and other animals is their ability to serve as precursors of vitamin A. Although the carotenoid β-carotene possesses the greatest provitamin A activity because of its two β-ionone rings (Figure 9.16), other commonly

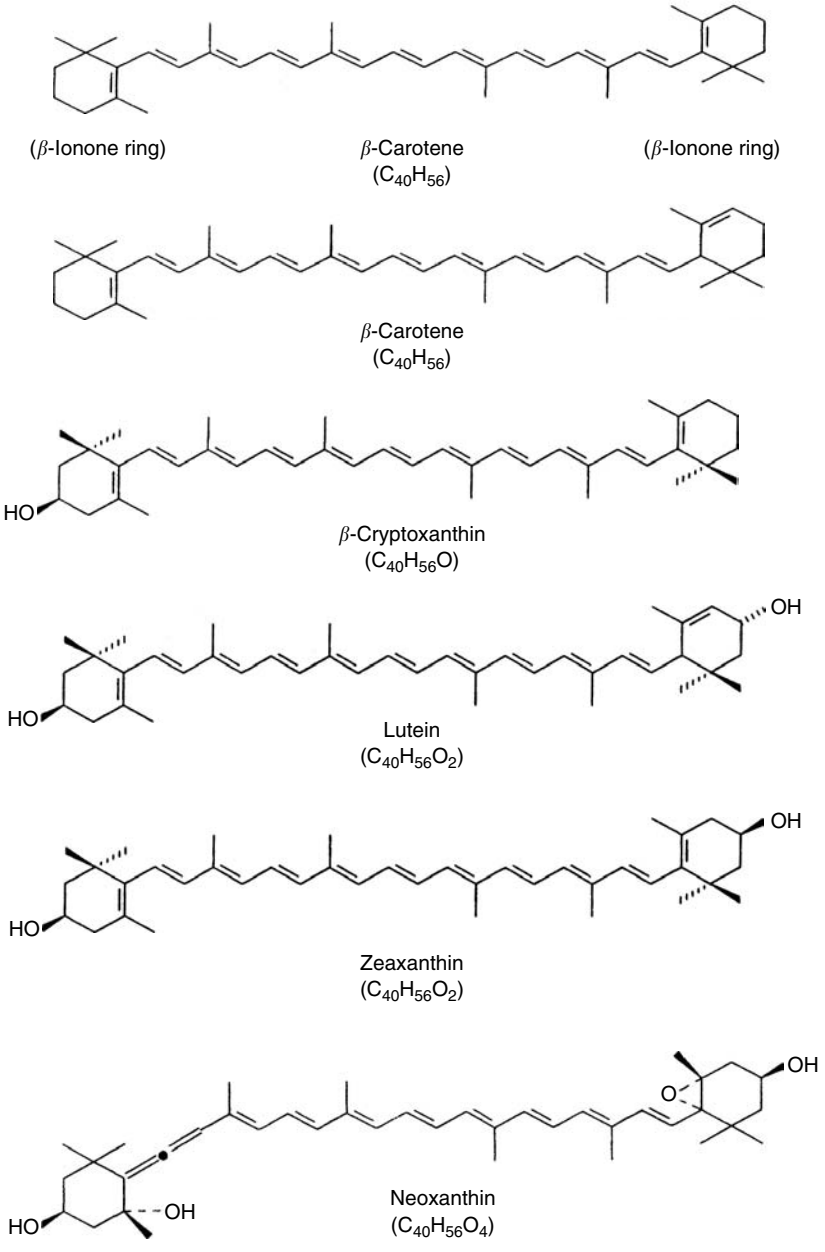


FIGURE 9.16 Structures of commonly occurring carotenoids. Lutein (yellow) from green leaves, corn, and marigold; zeaxanthin (yellow) from corn and saffron; β-cryptoxanthin (yellow) from corn; β-carotene (yellow) from carrots; β-carotene (yellow) from carrots and sweet potatoes; neoxanthin (yellow) from green leaves; capsanthin (red) from red peppers; violaxanthin (yellow) from green leaves; and bixin (yellow) from annatto seeds.

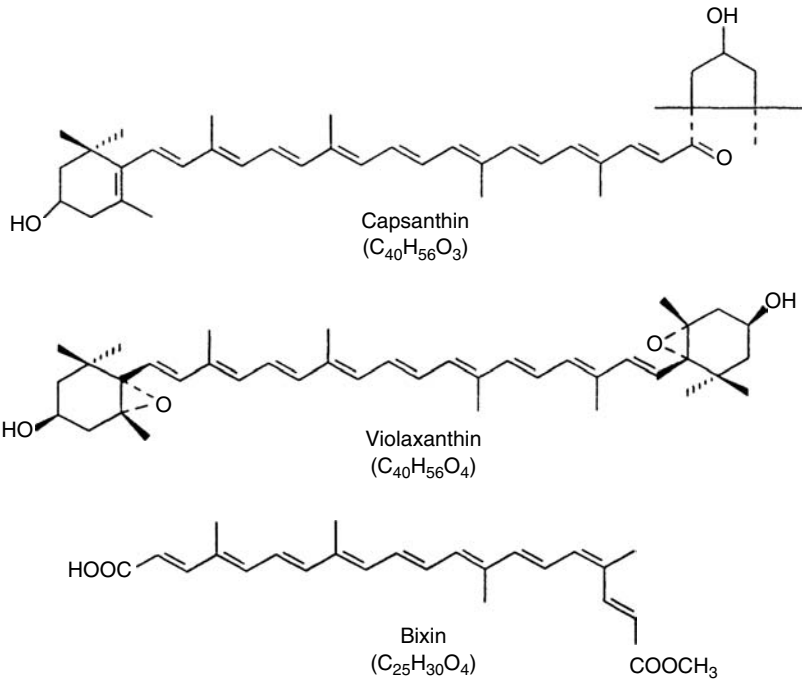


FIGURE 9.16 Continued.

consumed carotenoids, such as α-carotene and β-cryptoxanthin, also possess provitamin A activity. Provitamin A carotenoids present in fruits and vegetables are estimated to provide 30–100% of the vitamin A requirement in human populations. A prerequisite to vitamin A activity is the existence of the retinoid structure (with the β-ionone ring) in the carotenoid. Thus, only a few carotenoids possess vitamin activity. This topic is covered thoroughly in Chapter 7.

In 1981, Peto et al. [111] drew attention to these pigments because of their epidemiological findings that consumption of fruits and vegetables high in carotenoid content is associated with a decreased incidence of specific cancers in humans. More recently, interest has focused on the presence of carotenoids in the diet and on their physiological significance. These findings have stimulated a substantial increase in carotenoid research. An overview on the impact of carotenoids on health and disease can be found in Krinsky et al. [79].

9.2.3.1 Structures of Carotenoids

Carotenoids are comprised of two structural groups: the hydrocarbon carotenes and the oxygenated xanthophylls. Oxygenated carotenoids (xanthophylls) consist of a variety of derivatives frequently containing hydroxyl, epoxy, aldehyde, and keto groups. In addition, fatty acid esters of hydroxylated carotenoids are also widely found in nature. Thus, over 700 carotenoid structures have been identified and compiled [14]. Furthermore, when geometric isomers of *cis* (*Z*) or *trans* (*E*) forms are considered, a great many more configurations are possible.

The basic carotenoid structural backbone consists of isoprene units linked covalently in either a head-to-tail or a tail-to-tail fashion to create a symmetrical molecule (Figure 9.17). Other carotenoids are derived from this primary structure of 40 carbons. Some structures contain cyclic end groups (β-carotene, Figure 9.16) while others possess either one or no cyclization (lycopene, the prominent red pigment in tomatoes) (Figure 9.17). Other carotenoids may have shorter carbon skeletons and are known as apocarotenals (such as bixin). Although rules exist for naming and numbering all carotenoids [66,67], the trivial names are commonly used and presented in this chapter.

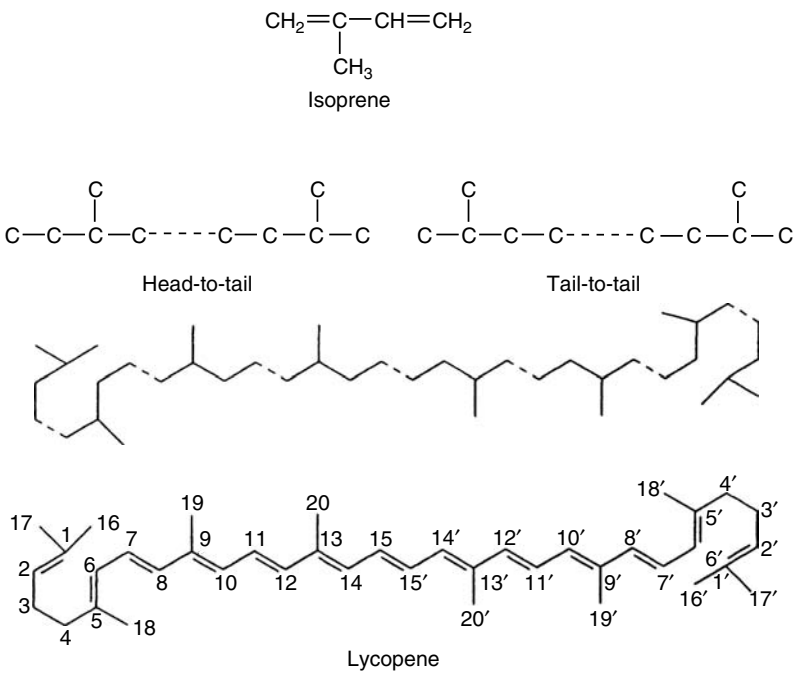


FIGURE 9.17 Joining of isoprenoid units to form lycopene (primary red pigment of tomatoes). (From Foote, C. S. (1968). *Science* 162:963–970.)

The most common carotenoid found in plant tissues is β-carotene. This carotenoid is also used as a colorant in foods. Both the naturally derived and synthetic forms can be added to food products. Some carotenoids found in plants are shown in Figure 9.16, and they include α-carotene (carrots), capsanthin (red peppers, paprika), lutein, a diol of α-carotene, its esters (marigold petals), and bixin (annatto seed). Other common carotenoids found in foods include zeaxanthin (a diol of β-carotene), violaxanthin (an epoxide carotenoid), neoxanthin (an allenic triol), and β-cryptoxanthin (a hydroxylated derivative of β-carotene).

Animals derive carotenoids pigments by consumption of carotenoid-containing plant materials. For example, the pink color of salmon flesh is due mainly to the presence of astaxanthin, which is obtained by ingestion of carotenoid-containing marine plants. It is also well known that some carotenoids in both plants and animals are bound to or associated with proteins. The red astaxanthin pigment of shrimp and lobster exoskeletons is blue in color when complexed with proteins. Heating denatures the complex and alters the spectroscopic and visual properties of the pigment, thus changing the color from blue to red. Other examples of carotenoid–chlorophyll–protein complexes are ovoverdin, the green pigment found in lobster eggs, and the carotenoid–chlorophyll–protein complexes in plant chloroplasts. Other unique structures include carotenoid glycosides, some of which are found in bacteria and other microorganisms. One example of a carotenoid glycoside present in plants is the carotenoid crocin found in saffron.

9.2.3.2 Occurrence and Distribution

Edible plant tissues contain a wide variety of carotenoids [55]. Red, yellow, and orange fruits, root crops, and vegetables are rich in carotenoids. Prominent examples include tomatoes (lycopene), carrots (α- and β-carotenes), red peppers (capsanthin), pumpkins (β-carotene), squashes (β-carotene), corn (lutein and zeaxanthin), and sweet potatoes (β-carotene). All green leafy vegetables contain carotenoids but their color is masked by the green chlorophylls. Generally, the largest concentrations

of carotenoids exist in those tissues with the largest amount of chlorophyll pigments. For example, spinach and kale are rich in carotenoids, and peas, green beans, and asparagus contain significant concentrations.

Many factors influence the carotenoid content of plants. In some fruits, ripening may bring about dramatic changes in carotenoids. For example, in tomatoes, the carotenoid content, especially lycopene, increases significantly during the ripening process. Thus, concentrations differ depending on the stage of plant maturity. Even after harvest, tomato carotenoids continue to be synthesized. Since light stimulates biosynthesis of carotenoids, the extent of light exposure is known to affect their concentration. Other factors that alter carotenoid occurrence or amount include growing climate, pesticide and fertilizer use, and soil type [55].

9.2.3.3 Physical Properties, Extraction, and Analysis

All classes of carotenoids (hydrocarbons: carotenes and lycopene and oxygenated xanthophylls) are lipophilic compounds and thus are soluble in oils and organic solvents [27]. They are moderately heat stable and are subject to loss of color by oxidation. Carotenoids can be easily isomerized by heat, acid, or light. Since they range in color from yellow to red, detection wavelengths for monitoring carotenoids typically range from approximately 430–480 nm. The higher wavelengths are usually used for some xanthophylls to prevent interference from chlorophylls. Many carotenoids exhibit spectral shifts after reaction with various reagents, and these spectral changes are useful to assist in identification.

The complex nature and diversity of carotenoid compounds present in plant foods necessitate chromatographic separation [119]. Extraction procedures for quantitative removal of carotenoids from tissue utilize organic solvents that must penetrate a hydrophilic matrix. Hexane–acetone mixtures are commonly employed for this purpose, but special solvents and treatments are sometimes needed to achieve satisfactory separation [75].

Many chromatographic procedures, including HPLC, have been developed for separating carotenoids [25,32,124,139]. Special analytical challenges occur when carotenoid esters, *cis*/*trans* isomers, and optical isomers need to be separated and identified.

9.2.3.4 Chemical Properties

9.2.3.4.1 Oxidation

Carotenoids are easily oxidized because of the large number of conjugated double bonds. Such reactions cause color loss of carotenoids in foods and are the major degradation mechanisms of concern. The stability of a particular pigment to oxidation is highly dependent on its environment. Within tissues, the pigments are often compartmentalized and protected from oxidation. However, physical damage to the tissue or extraction of the carotenoids increases their susceptibility to oxidation. Furthermore, storage of carotenoid pigments in organic solvents will often accelerate decomposition. Because of the highly conjugated, unsaturated structure of carotenoids, the products of their degradation are very complex. These products are largely uncharacterized, except for β-carotene. Shown in Figure 9.18 are various degradation products of β-carotene during oxidation and thermal treatments. During oxidation, epoxides and carbonyl compounds are initially formed. Further oxidation results in formation of short-chain mono- and dioxygenated compounds including epoxy-β-ionone. Generally, epoxides form mainly within the end rings, while oxidative scission can occur at a variety of sites along the chain. For provitamin A carotenoids, epoxide formation in the ring results in loss of the provitamin activity. Extensive autoxidation will result in bleaching of the carotenoid pigments and loss in color. Oxidative destruction of β-carotene is intensified in the presence of sulfite and metal ions [110].

Enzymatic activity, particularly lipoxygenase, hastens oxidative degradation of carotenoid pigments. This occurs by indirect mechanisms. Lipoxygenase first catalyzes oxidation of unsaturated

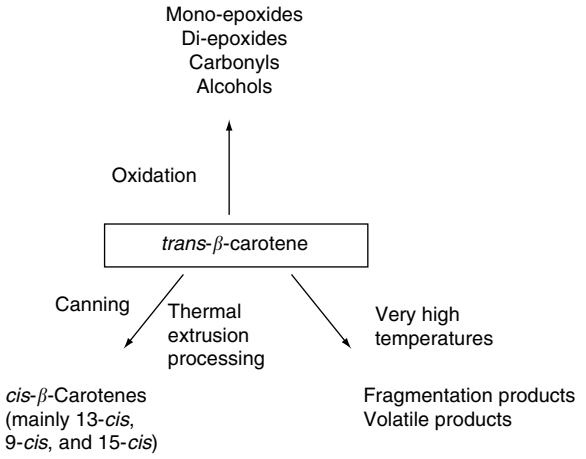


FIGURE 9.18 Degradation of all-*trans*-β-carotene.

or polyunsaturated fatty acids to produce peroxides, and these in turn react readily with carotenoid pigments. In fact, this coupled reaction scheme is quite efficient, and the loss of carotene color and decreased absorbance in solution are often used as an assay for lipoxygenase activity [8].

9.2.3.4.2 Antioxidative Activity

Because carotenoids can be readily oxidized, it is not surprising that they have antioxidant properties. In addition to cellular and *in vitro* protection against singlet oxygen, carotenoids, at low oxygen partial pressures, inhibit lipid peroxidation [17]. At high oxygen partial pressures, β-carotene has pro-oxidant properties [18]. In the presence of molecular oxygen, photosensitizers (i.e., chlorophyll), and light, singlet oxygen may be produced, which is a highly reactive oxygen species. Carotenoids are known to quench singlet oxygen and thereby protect against cellular oxidative damage. Not all carotenoids are equally effective as photochemical protectors. For example, lycopene is known to be especially efficient in quenching singlet oxygen relative to other carotenoid pigments [30,130].

It has been proposed that the antioxidant functions of carotenoids play a role in reducing the risk of cancer, cataracts, atherosclerosis, and the processes of aging [21]. A detailed overview on the antioxidant role of carotenoid compounds is beyond the scope of this discussion, and the reader is referred to several excellent reviews [18,47,78,104,153].

9.2.3.4.3 Cis/Trans Isomerization

In general, the conjugated double bonds of carotenoid compounds exist in an all-*trans* configuration. The *cis* isomers of a few carotenoids can be found naturally in plant tissues, especially in algae sources, which are currently being harvested as a source of carotenoid pigments. Isomerization reactions are readily induced by thermal treatments, exposure to organic solvents, contact for prolonged periods with certain active surfaces, treatment with acids, and illumination of solutions (particularly with iodine present). Iodine-catalyzed isomerization is a useful means in the study of photoisomerization because an equilibrium mixture of isomeric configurations is formed. Theoretically, large numbers of possible geometrical configurations could result from isomerization because of the extensive number of double bonds present in carotenoids. For example, β-carotene has potentially 272 different *cis* forms. However, because of steric contraints, only a limited number of *cis* isomers can occur in carotenoids. Because of the complexity of various *cis*/*trans* isomers within a single carotenoid, only recently have accurate methods been developed to study these compounds in foods [102,144]. *Cis*/*trans* isomerization affects the provitamin A activity but not the color of

carotenoids. The provitamin A activity of β-carotene *cis* isomers ranges, depending on the isomeric form, from 13 to 53% as compared to that of all-*trans*-β-carotene [154].

9.2.3.5 Stability During Processing

Carotenoids are relatively stable during typical storage and handling of most fruits and vegetables. Freezing causes little change in carotene content. However, blanching is known to influence the level of carotenoids. Often blanched plant products exhibit an apparent increase in carotenoid content relative to raw tissues. This is caused by inactivation of lipoxygenase, which is known to indirectly catalyze oxidative decomposition of carotenoids, the loss of soluble constituents into the blanch water, or the mild heat treatments traditionally used during blanching may enhance the efficiency of extraction of the pigments relative to fresh tissue. In addition, severe physical homogenization and thermal treatments also increase extraction [149] and bioavailability when consumed [114,143]. Lye peeling, which is commonly used for sweet potatoes, causes little destruction or isomerization of carotenoids.

Although carotene historically has been regarded as fairly stable during heating, it is now known that heat sterilization induces *cis*/*trans* isomerization reactions as shown in Figure 9.18. To lessen excessive isomerization, the severity of thermal treatments should be minimized when possible. In the case of extrusion cooking and high-temperature heating in oils, not only will carotenoids isomerize but also thermal degradation will also occur. Very high temperatures can yield fragmentation products that are volatile. Products arising from severe heating of β-carotene in the presence of air are similar to those arising from severe heating of β-carotene oxidation (Figure 9.18). In contrast, air dehydration exposes carotenoids to oxygen, which can cause extensive degradation of carotenoids. Dehydrated products that have large surface-to-mass ratios, such as carrot or sweet potato flakes, are especially susceptible to oxidative decomposition during drying and storage in air.

When *cis* isomers are created, only slight spectral shifts occur and thus color of the product is mostly unaffected; however, a decrease in provitamin A activity occurs. These reactions have important nutritional effects that should be considered when selecting analytical measurements for provitamin A. The older methods for vitamin A determination in foods did not account for the differences in the provitamin A activity of individual carotenoids or their isomeric forms. Therefore, older nutritional data for foods are in error, especially for foods that contain high proportions of provitamin A carotenoids other than β-carotene and those that contain a significant amount of *cis* isomers.

9.2.4 Anthocyanins and Other Phenols

9.2.4.1 Anthocyanins

Phenolic compounds comprise a large group of organic substances, and flavonoids are an important subgroup. The flavonoid subgroup contains the anthocyanins, one of the most broadly distributed pigment groups in the plant world. Anthocyanins are responsible for a wide range of colors in plants, including blue, purple, violet, magenta, red, and orange. The word anthocyanin is derived from two Greek words: anthos, flower, and kyanos, blue. These compounds have attracted the attention of chemists for years, two of the most notable investigators being Sir Robert Robinson (1886–1975) and Professor Richard Willstätter (1872–1942). Both were awarded Nobel prizes in chemistry, in part for their work with plant pigments.

9.2.4.1.1 Structure

Anthocyanins belong to the flavonoid group because of their characteristic $C_6C_3C_6$ carbon skeleton. The basic chemical structure of the flavonoid group and the relationship to anthocyanin are shown in Figure 9.19. Within each group there are many different compounds with their color depending on the presence and number of substituents attached to the molecule.

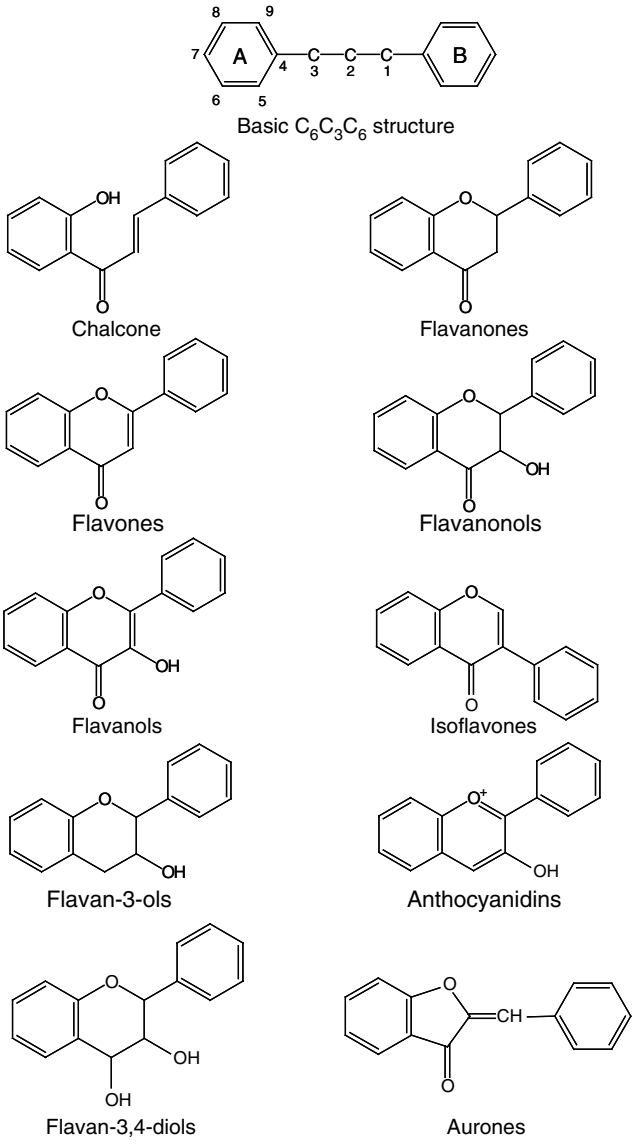


FIGURE 9.19 Carbon skeleton of some important flavonoids, classified by their C-3 chain structure.

The basic structure of anthocyanins is 2-phenylbenzopyrylium of flavylium salt (Figure 9.20). Anthocyanins exist as glycosides of polyhydroxy and/or polymethoxy derivatives of the salt. Anthocyanins differ in the number of hydroxyl and/or methoxy groups present, the types, numbers, sites of attachment of sugars to the molecule, and the types and numbers of aliphatic or aromatic acids that are attached to the sugars in the molecule. The most common sugars are glucose, rhamnose, galactose, arabinose, xylose, and homogenous or heterogeneous di- and trisaccharides formed as glycosides of these sugars. Acids most commonly involved in acylation of sugars are aromatic acids including *p*-coumaric, caffeic, ferulic, sinapic, gallic, or *p*-hydroxybenzoic acids, and/or aliphatic acids such as malonic, acetic, malic, succinic, or oxalic acids. These acyl substituents are commonly bound to the C-3 sugar, esterified to the 6-OH or less frequently to the 4-OH group of the sugars. However, anthocyanins containing rather complicated acylation patterns attached on different sugar moieties have been reported [128,140].

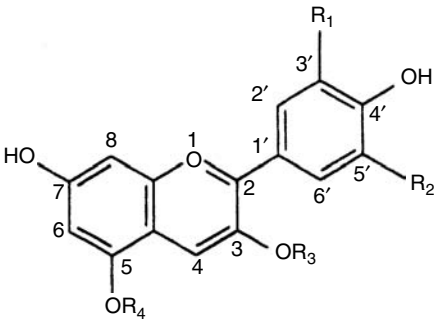


FIGURE 9.20 The flavylium cation. R_1 and R_2 = —H, —OH, or —OCH_3, R_3 = —glycosyl, R_4 = —H or —glycosyl.

When the sugar moiety of an anthocyanin is hydrolyzed, the aglycone (the nonsugar hydrolysis product) is referred to as an anthocyanidin. The color of anthocyanins and anthocyanidins results from excitation of a molecule by visible light. The ease with which a molecule is excited depends on the relative electron mobility in the structure. Double bonds, which are abundant in anthocyanins and anthocyanidins, are excited very easily, and their presence is essential for color. There are 19 naturally occurring anthocyanidins, but only six occur commonly in foods (Figure 9.21). It should be noted that increasing substitution on the anthocyanidin portion of the molecule results in a deeper hue. The deepening of hue is the result of a bathochromic shift (longer wavelength), which means that the light absorption band in the visible spectrum shifts from a shorter wavelength to a longer wavelength, with a resulting change in color from orange/red to purple/blue. An opposite change is referred to as a hypsochromic shift. Bathochromic effects are caused by auxochrome groups, groups that by themselves have no chromophoric properties but cause deepening in the hue when attached to the molecule. Auxochrome groups are electron-donating groups, and in the case of anthocyanidins they are the hydroxyl and methoxy groups. The methoxy groups, because their electron-donating capacity is greater than that of hydroxyl groups, cause a greater bathochromic shift than do hydroxyl groups. The effect of the number of methoxy groups on redness is illustrated in Figure 9.21. In anthocyanins, the type and number of sugar substitution and acylation also play an important role on the color characteristics, as well as several other factors, such as change in pH, metal complex formation, and copigmentation. These will be discussed later.

Anthocyanidins are less water soluble than their corresponding glycosides (anthocyanins) and they are not found free in nature. Those reported, other than the 3-deoxy form, which is yellow, are most likely hydrolysis products formed during isolation procedures. The free 3-hydroxyl group in the anthocyanidin molecule destabilizes the chromophore; therefore, the 3-hydroxyl group is always glycosylated. Additional glycosylation is most likely to occur at C-5 and can also occur at C-7, -3′, -4′, and/or -5′ hydroxyl group (Figure 9.20). Steric hindrance precludes glycosylation at both C-3′ and C-4′ [15].

With this structural diversity, it is not surprising that more than 600 different anthocyanins have been identified in the plant world [2]. Plants not only contain mixtures of anthocyanins but also the relative concentrations vary among cultivars and with maturity. Total anthocyanin content varies among plants and ranges from about 20 mg/100 g fresh weight to as high as a few grams per 100 g. The reader is referred to a book by Mazza and Miniati [97] for a more detailed account of anthocyanins in fruits, vegetables, and grains.

9.2.4.1.2 Color and Stability of Anthocyanins

Anthocyanin pigments are relatively unstable, with greatest stability occurring under acidic conditions. Both the color characteristics (hue and chroma) of the pigment and its stability are greatly

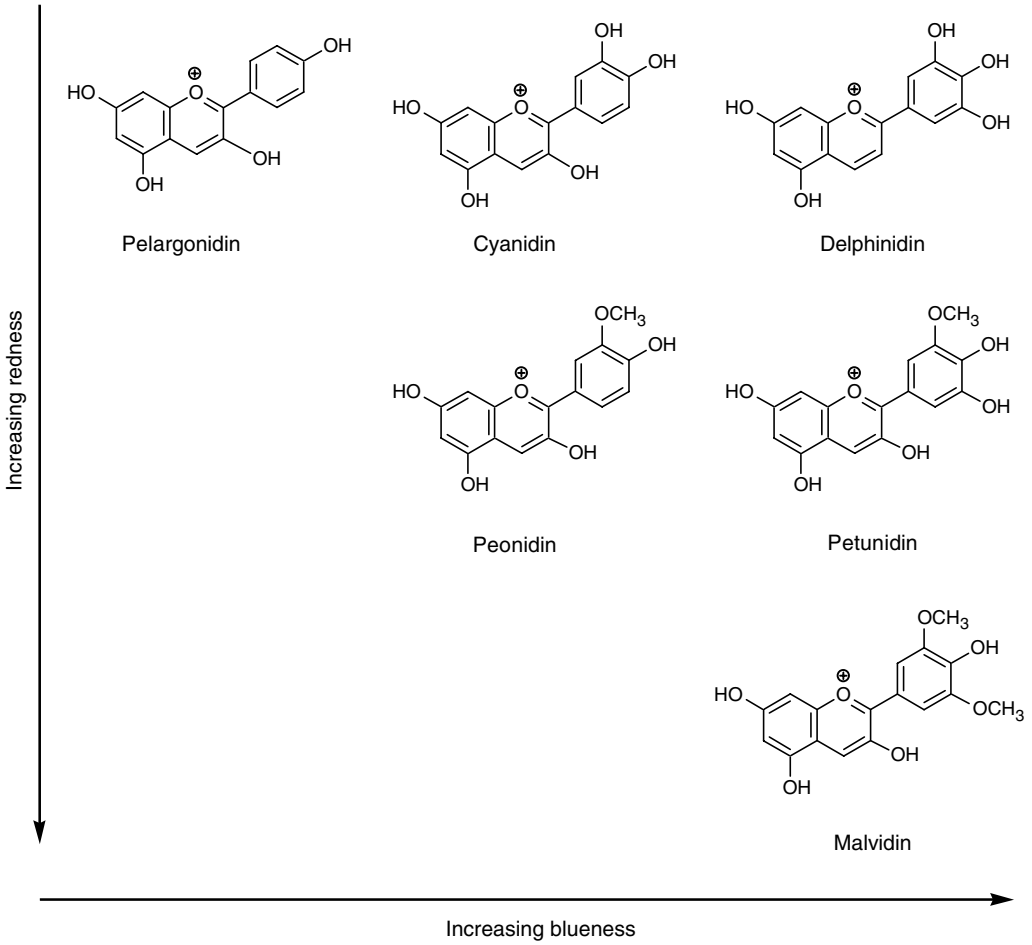


FIGURE 9.21 Most common anthocyanidins in foods, arranged in increasing redness and blueness.

impacted by substituents on the aglycone. Degradation of anthocyanins occurs not only during extraction from plant tissue but also during processing and storage of food tissues.

Knowledge of the chemistry of anthocyanins can be used to minimize degradation by proper selection of processes and by selection of anthocyanin pigments that are most suitable for the intended application. Major factors governing degradation of anthocyanins are pH, temperature, and oxygen concentration. Factors that are usually of less importance are the presence of degradative enzymes, ascorbic acid, sulfur dioxide, metal ions, and sugars. In addition, copigmentation may affect or appear to affect the degradation rate.

9.2.4.1.3 Structural Transformation and pH

Degradation rates vary greatly among anthocyanins because of their diverse structures. Generally, increased hydroxylation decreases stability, while increased methylation increases stability. The color of foods containing anthocyanins that are rich in pelargonidin, cyanidin, or delphinidin aglycones is less stable than that of foods containing anthocyanins that are rich in petunidin or malvidin aglycones. The increased stability of the latter group occurs because reactive hydroxyl groups are blocked. It follows that increased glycosylation, as in monoglucosides and diglucosides, increases stability. It has also been shown, although it is not fully understood why, that the type of sugar moiety influences stability. Starr and Francis [131] found that cranberry anthocyanins that contained galactose were

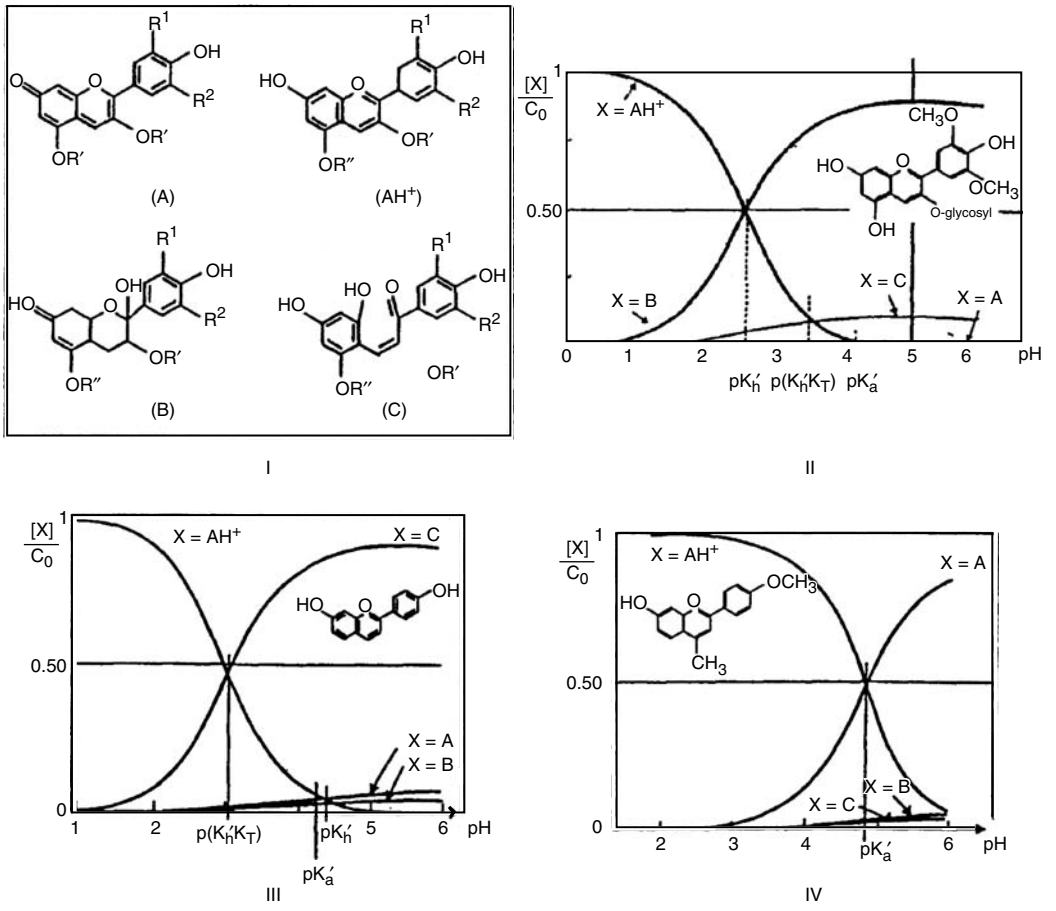


FIGURE 9.22 (I) The four anthocyanin structures present in aqueous acidic solution at room temperatures: A, quinonoidal base (blue); (AH^+) flavylium salt (red); B, pseudobase or carbinol (colorless); C, chalcone (colorless). (II–IV) Equilibrium distribution at 25°C of AH^+, A, B, and C as a function of pH: (II) for malvidin-3-glucoside; (III) for 4′,7-hydroxyflavylium chloride; (IV) for 4′-methoxyl-4-methyl-7-hydroxyflavylium chloride. (From Brouillard, R. (1982). In *Anthocyanins as Food Colors.* P. Markakis (Ed.). Academic Press, New York, pp. 1–40.)

more stable during storage than those containing arabinose. Cyanidin 3-(2 glucosylrutinoside) at pH 3.5, 50°C, has a half-life of 26 h compared to 16 h for cyanidin-3-rutinoside [146]. These examples illustrate that substituents have a marked effect on anthocyanin stability although they themselves do not react.

In an aqueous medium, including foods, anthocyanins can exist in four possible structural forms depending on pH (Figure 9.22, I): the blue quinonoidal base (A), the red flavylium cation (AH^+), the colorless carbinol pseudobase (B), and the colorless chalcone (C). Shown in Figure 9.22 are the equilibrium distributions of these four forms in the pH range 0–6 for malvidin-3-glucoside (Figure 9.22, II), dihydroxyflavylium chloride (Figure 9.22, III), and 4′-methoxy-4-methyl-7-hydroxyflavylium chloride (Figure 9.22, IV). For each pigment only two of the four species are important over this pH range. In a solution of malvidin-3-glucoside at low pH the flavylium structure dominates, while at pH 4–6 the colorless carbinol dominates. A similar situation exists with 4′,7-hydroxyflavylium except the equilibrium mixture consists mainly of the flavylium and the chalcone structure. Thus, as the pH approaches 6 the solution becomes colorless. In a solution of 4′-methoxyl-4-methyl-7-hydroxyflavylium chloride, an equilibrium exists between the flavylium cation and the quinonoidal

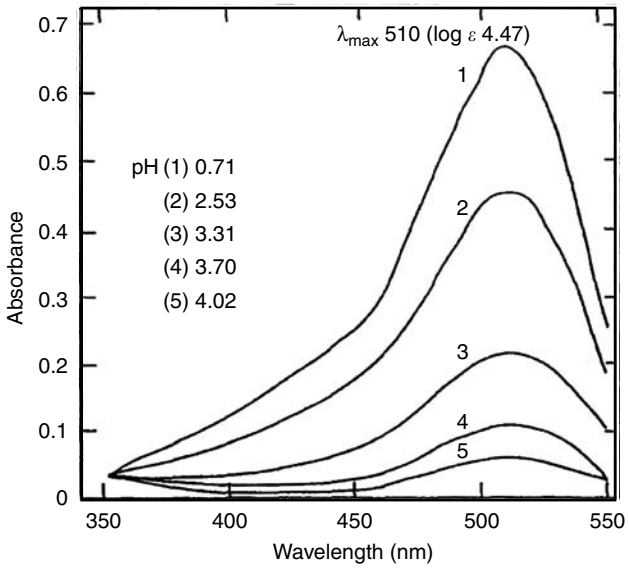


FIGURE 9.23 Absorption spectra of cyanidin-3-rhamnosylglucoside in buffer solutions at pH 0.71–4.02. Pigment concentration 1.6×10^{-2} g/L. (From Falk, J. E. and J. N. Phillips (1964). In *Chelating Agents and Metal Chelates*. F. P. Dwyer and D. P. Mellor (Eds.). Academic Press, New York, pp. 441–490.)

base. This solution therefore is colored throughout the pH 0–6 range, turning from red to blue as pH is increased in this range.

To further demonstrate the effect of pH on the color of anthocyanins, the spectra for cyanidin-3-rhamnosylglucoside in buffer solutions at pH levels between 0.71 and 4.02 are shown in Figure 9.23. Although the absorption maximum remains the same over this pH range, the intensity of absorption decreases with increasing pH. Color changes in a mixture of cranberry anthocyanins as a function of pH are shown in Figure 9.24. In aqueous medium, as in cranberry cocktail, changes in pH can cause major changes in color. Anthocyanins show their greatest tinctorial strength at approximately pH 1.0, when the pigment molecules are mostly in the ionized form. At pH 4.5, anthocyanins in fruit juices are nearly colorless (slightly bluish) if yellow flavonoids are not present. If yellow pigments are present, as is common in fruits, the juice will be green.

9.2.4.1.4 Temperature

Anthocyanin stability in foods is greatly affected by temperature. Rates of degradation are also influenced by the presence or absence of oxygen and, as already pointed out, by pH and structural conformation. In general, structural features that lead to increased pH stability also lead to increased thermal stability. Highly hydroxylated anthocyanidins are less stable than methylated, glycosylated, or acylated anthocyanidins. For example, the half-life of 3,4′,5,5′,7-pentahydroxyflavylium at pH 2.8 is 0.5 day compared to 6 days for the 3,4′,5,5′,7-pentamethoxyflavylium [97]. Under similar conditions, the half-life for cyanidin-3-rutinoside is 65 days compared to 12 h for cyanidin [95]. It should be noted that comparison of published data for pigment stability is difficult because of differing experimental conditions used. One of the errors in published data involves a failure to consider the equilibrium reactions among the four known anthocyanin structures (Figure 9.22, I).

Heating shifts the equilibria toward the chalcone and the reverse reaction is slower than the forward reaction. It takes, for example, 12 h for the chalcone of a 3,5-diglycoside to reach equilibrium. Since determination of the amount of pigment remaining is generally based on measurement of the flavylium salt, an error is introduced if insufficient time is allowed for equilibrium to be attained [95].

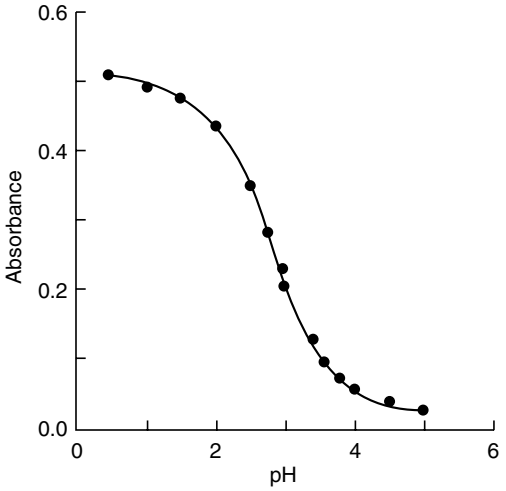


FIGURE 9.24 Absorbance of cranberry anthocyanins vs. pH. (Adapted from Falk, J. E. and J. N. Phillips (1964). In *Chelating Agents and Metal Chelates*. F. P. Dwyer and D. P. Mellor (Eds.). Academic Press, New York, pp. 441–490.)

The exact mechanism of thermal degradation of anthocyanin has not been fully elucidated. Three pathways have been suggested. Coumarin 3,5-diglycoside is a common degradation product for anthocyanidin (cyanidin, peonidin, delphinidin, petunidin, and malvidin) 3,5-diglycoside (Figure 9.25). In path (a) the flavylium cation is first transformed to the quinonoidal base, then to several intermediates, and finally to the coumarin derivative and a compound corresponding to the B-ring. In path (b) (Figure 9.25) the flavylium cation is first transformed to the colorless carbinol base, then to the chalcone, and finally to brown degradation products. Path (c) (Figure 9.25) is similar except degradation products of chalcone are first inserted. These three proposed mechanisms suggest that thermal degradation of anthocyanins depends on the type of anthocyanin involved and the degradation temperature.

9.2.4.1.5 Oxygen and Ascorbic Acid

The unsaturated nature of the anthocyanidin structure makes it susceptible to molecular oxygen. It has been known for many years that when grape juice is hot-filled into bottles, complete filling of the bottles will delay degradation of the color from purple to dull brown. Similar observations have been made with other anthocyanin-containing juices. The positive effect of oxygen removal on retention of anthocyanin color has been further demonstrated by processing anthocyanin-pigmented fruit juices under nitrogen or vacuum [26,131]. Also, the stability of pigments from Concord grape juice in a dry beverage is greatly enhanced when the product is packaged in a nitrogen atmosphere.Though little information exists relating a_w to anthocyanin stability, stability was found to be greatest at a_w values in the range of 0.63–0.79 (Table 9.8).

It is known that ascorbic acid and anthocyanins disappear simultaneously in fruit juices, suggesting some direct interaction between the two molecules. This, however, has been discounted, and it is believed instead that ascorbic acid-induced degradation of anthocyanin results indirectly from hydrogen peroxide that forms during oxidation of ascorbic acid [68]. The latter reaction is accelerated by the presence of copper and inhibited by the presence of flavonols such as quercetin and quercitrin [129]. Conditions that do not favor formation of H_2O_2 during oxidation of ascorbic acid, therefore, account for anthocyanin stability in some fruit juices. H_2O_2 cleavage of the pyrylium ring by a nucleophilic attack at the C-2 position of the anthocyanin produces colorless esters and coumarin derivatives. These breakdown products may further degrade or polymerize and ultimately lead to a brown precipitate that is often observed in fruit juices.

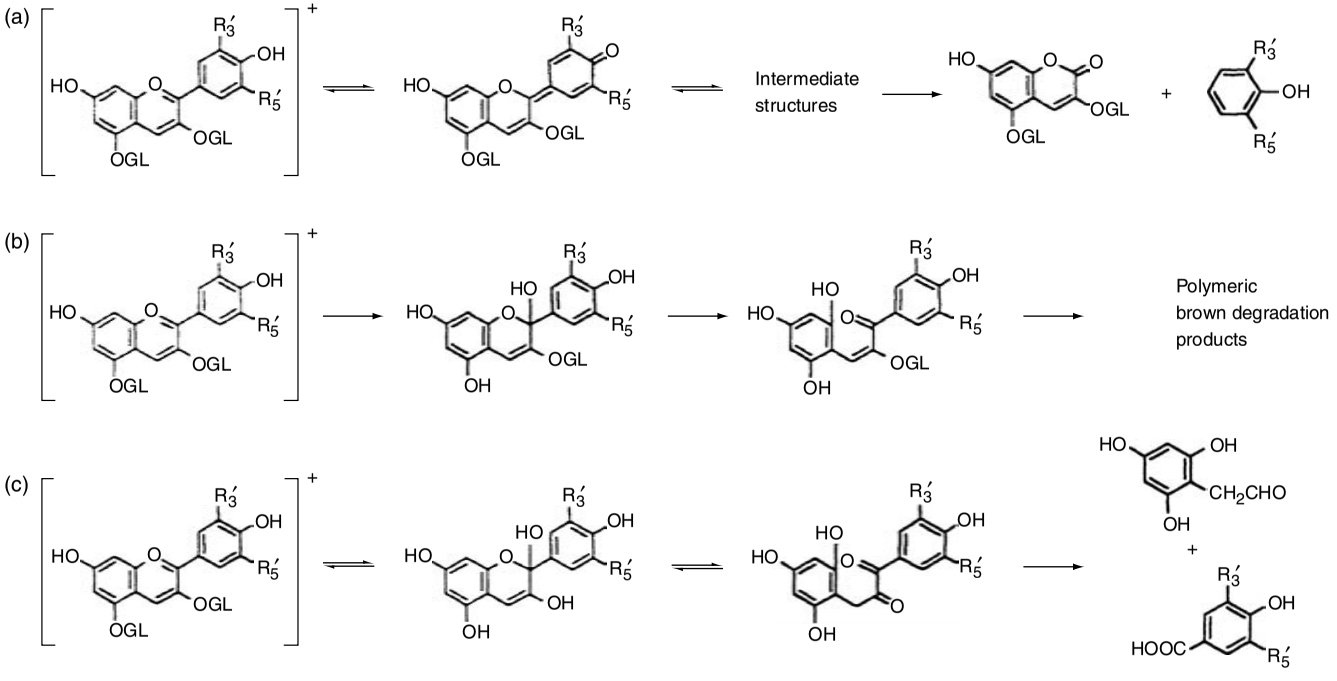


FIGURE 9.25 Mechanisms of degradation for anthocyanidin 3,5-diglycoside and for anthocyanidin 3-diglucosides. $R^{3'}$, $R^{5'}$ = −OH, −H, $-OCH_3$ or −OGL; GL = glucosyl group. (From Fulcrand, H., et al. (1998). *Phytochem.* 47:1401–1407.)

TABLE 9.8
Effect of a_w on Color Stability of Anthocyanins[a] During Heating as Measured by Absorbance

	Absorbance at Water Activities						
Holding Time at 43°C (min)	**1.00**	**0.95**	**0.87**	**0.74**	**0.63**	**0.47**	**0.37**
0	0.84	0.85	0.86	0.91	0.92	0.96	1.03
60	0.78	0.82	0.82	0.88	0.88	0.89	0.90
90	0.76	0.81	0.81	0.85	0.86	0.87	0.89
160	0.74	0.76	0.78	0.84	0.85	0.86	0.87
Percent change in absorbance (0–160 min)	11.9	10.5	9.3	7.6	7.6	10.4	15.5

[a] Concentration 700 mg/100 mL (1 g commercially dried pigment powder).

Source: Adapted from Govindarajan, S., et al. (1977). *J. Food Sci.* 42:571–577, 582.

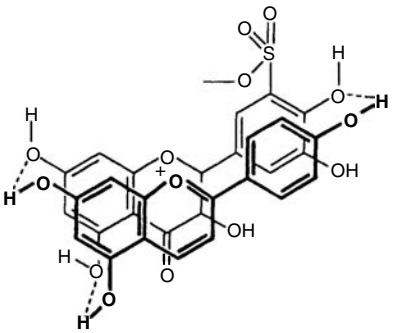


FIGURE 9.26 Molecular complex between anthocyanin and a polyhydroxyflavone sulfonate. (From Sweeny, J. G., et al. (1981). *J. Agric. Food Chem*. 29:563–567.)

9.2.4.1.6 Light

It is generally recognized that light accelerates degradation of anthocyanins. This adverse effect has been demonstrated in several fruit juices and red wine. In wine it has been determined that acylated and methylated diglycosides are more stable than nonacylated diglycosides, which are more stable than monoglycosides [145]. Copigmentation (anthocyanin condensation with themselves or other organic compound) can either accelerate or retard degradation, depending on the circumstances. Polyhydroxylated flavone, isoflavone, and aurone sulfonates exert a protective effect against photodegradation [136]. The protective effect is attributable to the formation of intermolecular ring interactions between the negatively charged sulfonate and the positively charged flavylium ion (Figure 9.26). Anthocyanins substituted at the C-5 hydroxyl groups are more susceptible to photodegradation than those unsubstituted at this position. Unsubstituted or monosubstituted anthocyanins are susceptible to nucleophilic attack at the C-2 and/or C-4 positions. Other forms of radiant energy such as ionizing radiation can also result in anthocyanin degradation [96].

9.2.4.1.7 Sugars and Their Degradation Products

Sugars at high concentrations, as in fruit preserves, stabilize anthocyanins. This effect is believed to result from a lowering of water activity (see Table 9.8). Nucleophilic attack of the flavylium cation

by water occurs at the C-2 position, forming the colorless carbinol base. When sugars are present at concentrations sufficiently low to have little effect on a_w, they or their degradation products sometimes can accelerate anthocyanin degradation. At low concentrations, fructose, arabinose, lactose, and sorbose have a greater degradative effect on anthocyanins than do glucose, sucrose, and maltose. The rate of anthocyanin degradation follows the rate of sugar degradation to furfural. Furfural that is derived from aldo-pentoses, and hydroxylmethylfurfural that is derived from keto-hexoses, result from the Maillard reaction or from oxidation of ascorbic acid. These compounds readily condense with anthocyanin, forming brown compounds. The mechanism of this reaction is unknown. The reaction is very temperature-dependent, is hastened by the presence of oxygen, and is very noticeable in fruit juices.

9.2.4.1.8 Metals

Metal complexes of anthocyanin are common in the plant world and they extend the color spectrum of flowers. Coated metal cans have long been found to be essential for retaining typical colors of anthocyanins of fruits and vegetables during sterilization in metal cans. Anthocyanins with vicinal, phenolic hydroxyl groups can sequester several multivalent metals. Complexation produces a bathochromic shift toward the blue. Addition of $AlCl_3$ to anthocyanin solutions has been used as an analytical tool to differentiate cyanidin, petunidin, and delphinidin from pelargonidin, peonidin, and malvidin. The latter group of anthocyanidins does not possess vicinal phenolic hydroxyls and will not react with Al^{3+} (Figure 9.21). Some studies have shown that metal complexation stabilizes the color of anthocyanin-containing foods. Ca, Fe, Al, and Sn ions have been shown to offer some protection to anthocyanins in cranberry juice; however, the blue and brown discoloration produced by tannin–metal complexes negates any beneficial effect [46].

A fruit discoloration problem referred to as "pinking" has been attributed to formation of metal anthocyanin complexes. This type of discoloration has been reported in pears, peaches, and lychees. It is generally believed that pinking is caused by heat-induced conversion of colorless proanthocyanidins to anthocyanins under acid conditions, followed by complex formation with metals [89].

9.2.4.1.9 Sulfur Dioxide

One step in the production of maraschino, candied and glacé cherries involves bleaching of anthocyanins by SO_2 at high concentrations (0.8–1.5%). Fruits containing anthocyanins are preserved by holding them in a solution containing 500–2000 ppm SO_2, resulting in the formation of a colorless complex. This reaction has been extensively studied, and it is believed that the reaction involves attachment of SO_2 at position C-4 (Figure 9.27). The reason for suggesting involvement of the 4 position is that SO_2 in this position disrupts the conjugated double bond system, which results in loss of color. The rate constant (k) for the discoloration reaction of cyanidin 3-glucoside has been calculated as 25,700/μA.

The large rate constant means that a small amount of SO_2 can quickly decolorize a significant amount of anthocyanin. Anthocyanins that are resistant to SO_2 bleaching either have the C-4 position

FIGURE 9.27 Colorless anthocyanin-sulfate (−SO_2) complex.

blocked or exist as dimers linked through their 4 position [13]. The bleaching that occurs during production of maraschino or candied cherries is irreversible.

9.2.4.1.10 Copigmentation

Anthocyanins are known to condense with themselves (self-association) and other organic compounds (copigmentation). Weak complexes form with proteins, tannins, other flavonoids, and polysaccharides. Although most of these compounds themselves are not colored, they augment the color of anthocyanins by causing bathochromic shifts and increased light absorption at the wavelength of maximum light absorption. These complexes also tend to be more stable during processing and storage. During winemaking, anthocyanins undergo a series of reactions to form more stable, complex wine pigments. The stable color of wine is believed to result from covalent self-association of anthocyanin. Such polymers are less pH sensitive and, because the association occurs through the four position, are resistant to discoloration by SO_2. In addition, anthocyanin-derived pigments (vitisin A and B) have been found in wine [6,48] as a result of the reaction between malvidin and pyruvic acid or acetaldehyde, respectively. This reaction causes an hypsochromic shift on its visible wavelength of absorption, producing a more orange/red hue as compared to the typical bluish purple of malvidin. However, the contribution of vitisin to total wine color may be minor [123].

Adsorption of the flavylium cation and/or the quinonoidal base to a suitable substrate, such as pectins or starches, can stabilize anthocyanins. This stabilization should enhance their utility as potential food color additives. Other condensation reactions can lead to color loss. Certain nucleophiles, such as amino acids, phloroglucinol, and catechin, can condense with flavylium cations to yield colorless 4-substituted flav-2-enes [95]. Proposed structures are shown in Figure 9.28.

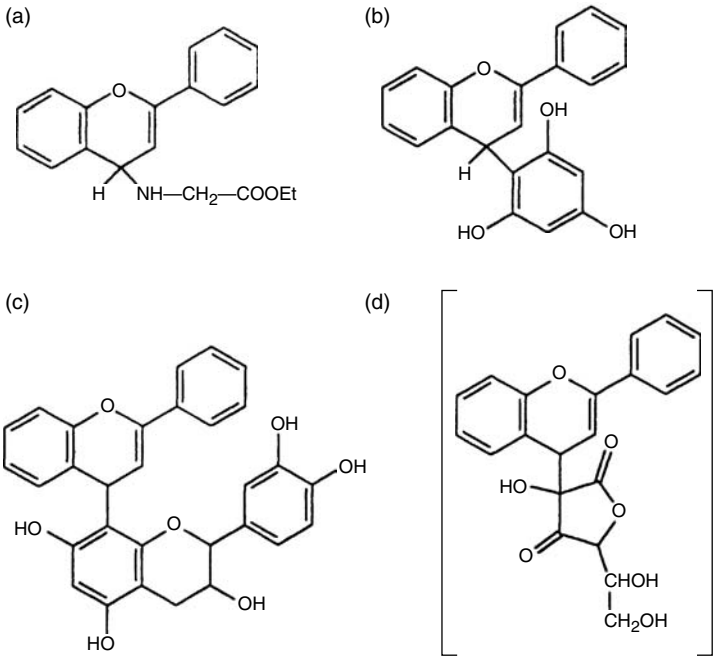


FIGURE 9.28 Colorless 4-substituted flav-2-enes resulting from the condensation of flavylium with (a) ethylglycine, (b) phloroglucinol, (c) catechin, and (d) ascorbic acid. (From Markakis, P. (1982). In *Anthocyanins as Food Colors*. P. Markakis (Ed.). Academic Press, New York, pp. 163–180.)

9.2.4.1.11 Acylated Anthocyanins as Natural Food Colorants

Discovery of acylated anthocyanins with high stability have raised the possibility that these pigments may impart desirable color and stability for commercial food products [50]. Examples of edible sources of such anthocyanins with desirable color and stability are radishes, red potatoes, red cabbage, black carrots, purple corn, and purple sweet potatoes. Among these, radishes and red potatoes stand out as potential alternatives for the use of FD&C Red No. 40 (allura red). Typical applications would be juices or water-based systems with pH below 3. However, other foods have been successfully colored with anthocyanin-based colorants; maraschino cherries (pH 3.5) with bright attractive and stable red color obtained with radish extract [51] were extremely close to those treated with allura red. Other potential applications for acylated anthocyanins include other challenging systems such as dairy products [42,50], including yogurt and milk. The unusual 3-deoxyanthocyanins from sorghum are also being investigated as potential alternatives to the use of artificial colorants [4]. These pigments are significantly more stable to pH changes, storage, and processing conditions, and provide colors ranging from yellow-orange to red. The increased stability of these pigments together with their added value due to their potential beneficial health effects provides new opportunities for their use in a variety of food applications.

9.2.4.1.12 Enzyme Reactions

Enzymes have been implicated in the decolorization of anthocyanins. Two groups have been identified: glycosidases and polyphenol oxidases. Together they are generally referred to as anthocyanases. Glycosidases, as the name implies, hydrolyze glycosidic linkages, yielding sugars(s) and the agylcone. Loss of color intensity results from the decreased solubility of the anthocyanidins and their transformation to colorless products. Polyphenol oxidases act in the presence of *o*-diphenols and oxygen to oxidize anthocyanins. The enzyme first oxidizes the *o*-diphenol to *o*-benzoquinone that in turn reacts with the anthocyanins by a nonenzymatic mechanism to form oxidized anthocyanins and degradation products (Figure 9.29) [95].

Although blanching of fruits is not a general practice, anthocyanin destroying enzymes can be inactivated by a short blanch treatment (45–60 s at 90–100°C). This has been suggested for sour cherries before freezing. Very low concentrations of SO_2 (30 ppm) have been reported to inhibit enzymatic degradation of anthocyanin in cherries [52]. Similarly, a heat-stabilization effect on anthocyanin has been noted when Na_2SO_3 is present [1].

9.2.4.2 Other Flavonoids

Anthocyanins, as previously mentioned, are the most prevalent flavonoids. Although most yellow colors in food are attributable to the presence of carotenoids, some are attributable to the presence of nonanthocyanin-type flavonoids. In addition, flavonoids also account for some of the whiteness of plant materials, and the oxidation products of those containing phenolic groups contribute to the browns and blacks found in nature. The term anthoxanthin (Greek words: anthos, flower; xanthos,

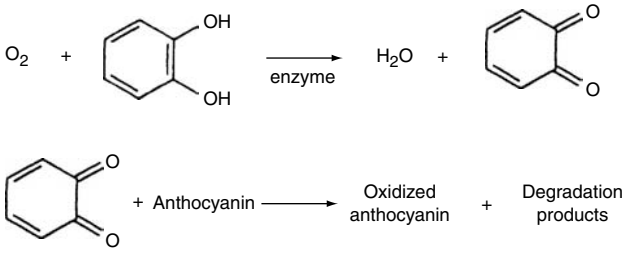


FIGURE 9.29 Proposed mechanisms of anthocyanin degradation by polyphenol oxidase. (From Peng, C. Y. and P. Markakis (1963). *Nature (Lond.)* 199:597–598.)

yellow) is also sometimes used to designate certain groups of yellow flavonoids. Differences among classes of flavonoids relate to the state of oxidation of the 3-carbon link (Figure 9.19). Structures commonly found in nature vary from flavan-3-ols (catechin) to flavonols (3-hydroxyflavones) and anthocyanins. The flavonoids also include flavanone, flavononols or dihydroflavonol, and flavan-3,4-diols (proanthocyanidin). In addition, there are five classes of compounds that do not possess the basic flavonoid skeleton, but are chemically related, and therefore are generally included in the flavonoid group. These are the dihydrochalcones, chalcones, isoflavones, neoflavones, and aurones. Individual compounds within this group are distinguished, as with anthocyanins, by the number of hydroxyl, methoxyl, and other substituents on the two benzene rings. Many flavonoid compounds carry a name related to the first source from which they were isolated, rather than being named according to the substituents of the respective aglycone. This inconsistent nomenclature has brought about confusion in assigning compounds to various classes.

9.2.4.2.1 Physical Properties

The light absorption characteristics of flavonoid classes clearly demonstrates the relationship of color and unsaturation within a molecule and the impact of auxochromes (groups present in a molecule that deepens the color). In the hydroxy-substituted flavans catechin and proanthocyanin, the unsaturation is interrupted between the two benzene rings, and therefore, the light absorption is similar to that of phenols, which exhibit maximum light absorption between 275 and 280 nm (Figure 9.30a).

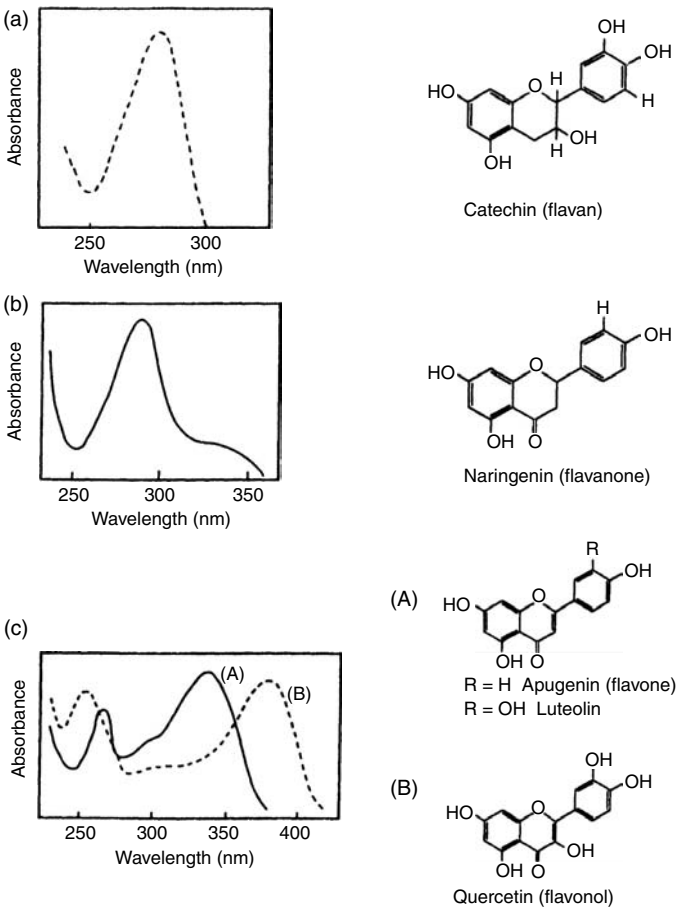


FIGURE 9.30 Absorption spectra of specific flavonoids.

In the flavanone naringenin, the hydroxyl groups only occur in conjunction with the carbonyl group at C-4, and therefore do not exert their auxochromic characteristics (Figure 9.30b). Therefore its light absorption is similar to that of flavans. In the case of the flavone luteolin (Figure 9.30c), the hydroxyl groups associated with both benzene rings exert their auxochormic characteristics through the conjugation of C-4. Light absorption of longer wavelength (350 nm) is associated with the B-ring, while that of shorter wavelength is associated with the A-ring. The hydroxyl group at C-3 in the flavonol quercetin causes a further shift to a still longer wavelength (380 nm) for maximum light absorption, compared to that of the flavones (Figure 9.30c). The flavonols, therefore, appear yellow if present at high enough concentration. Acylation and/or glycosylation results in further shifts in light absorption characteristics.

As previously mentioned, flavonoids of these types can become involved in copigmentation, and this occurrence has a major impact on many hues in nature. In addition, flavonoids, like anthocyanins, are chelators of metals. Chelation with iron or aluminum increases the yellow saturation. Luteolin when chelated with aluminum forms an attractive yellow color (390 nm).

9.2.4.2.2 Importance in Foods

Nonanthocyanin (NA) flavonoids make some contribution to color in foods; however, the paleness of most NA-flavonoids generally restricts their overall contribution. The whiteness of vegetables such as cauliflower, onion, and potato is attributable largely to NA-flavonoids, but their contribution to color through copigmentation is more important. The chelation characteristics of these compounds can contribute both positively or negatively to the color of foods. For example, rutin (3-rutinoside of quercetin) causes a greenish-black discoloration in canned asparagus when it complexes with ferric-state iron. The addition of a chelator such as ethylenediaminetetraacetic acid (EDTA) will inhibit this undesirable color. The tin complex of rutin has a very attractive yellow color, which contributed greatly to the acceptance of yellow wax beans until the practice of canning wax beans in plain tin cans was eliminated. The tin–rutin complex is more stable than the iron complex; thus the addition or availability of only very small amounts of tin would favor formation of the tin complex.

The color of black ripe olives is due in part to the oxidative products of flavonoids. One of the flavonoids involved is luteolin 7-glucoside. Oxidation of this compound and formation of the black color occur during fermentation and subsequent storage [11]. Other very important functions of flavonoids in foods are their antioxidant properties and their contribution to flavors, particularly bitterness.

9.2.4.2.3 Proanthocyanidins

Consideration of proanthocyanidins under the general topic of anthocyanins is appropriate. Although these compounds are colorless they have structural similarities with anthocyanidins. They can be converted to colored products during food processing. Proanthocyanidins are also referred to as leucoanthocyanidins or leucoanthocyanins. Other terms used to describe these colorless compounds are anthoxanthin, anthocyanogens, flavolans, flavylans, and flaylogens. The term leucoanthocyanidin is appropriate if it is used to designate the monomeric flaven-3,4-diol (Figure 9.31) that which is the basic building block of proanthocyanidins. The latter can occur as dimers, trimers, or higher polymers. The intermonomer linkage is generally through carbons C-4 and C-8 or C-4 and C-6.

Proanthocyanidins were first found in cocoa beans, where upon heating under acidic conditions they hydrolzye into cyanidin and (−)-epicatechin (Figure 9.32) [41]. Dimeric proanthocyanidins have been found in apples, pears, kola nuts, and other fruits. These compounds are known to degrade in air or under light to red-brown stable derivatives. They contribute significantly to the color of apple juice and other fruit juices, and to astringency in some foods. To produce astringency, proanthocyanidins of two to eight units interact with proteins. Other proanthocyanidins found in nature will yield on hydrolysis the common anthocyanidins—pelargonidin, petunidin, or delphinidin.

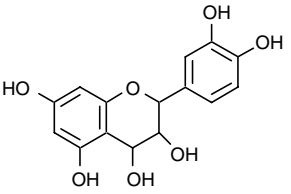


FIGURE 9.31 Basic structure of proanthocyanidin.

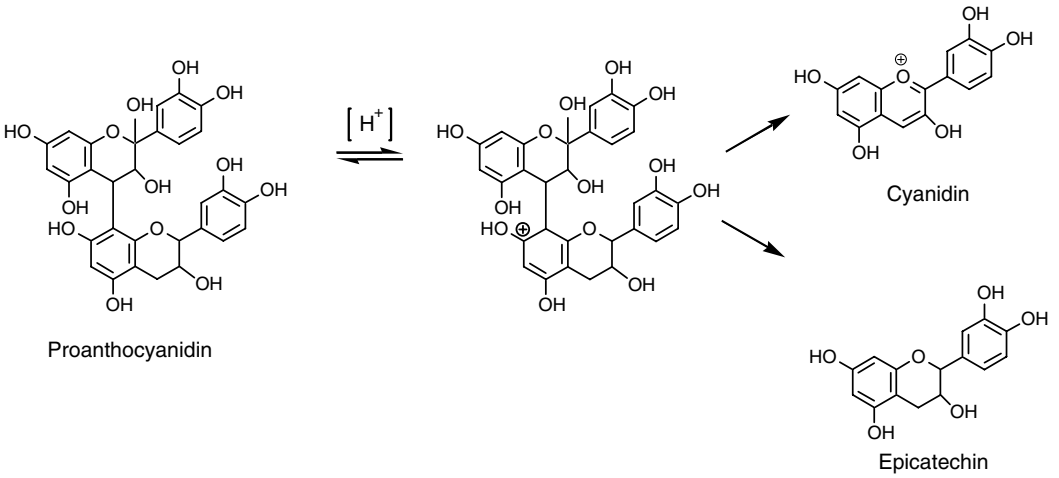


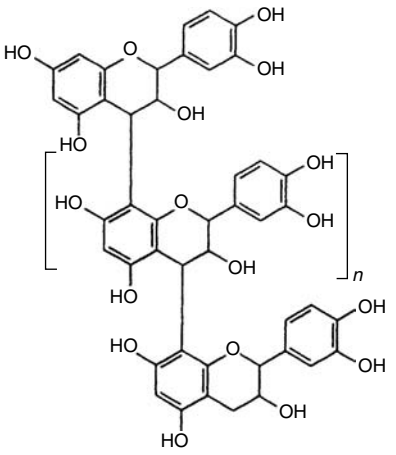
FIGURE 9.32 Mechanism of acid hydrolysis of proanthocyanidin. (From Forsyth, W. G. C. and J. B. Roberts (1958). *Chem. Ind. (Lond.)* 755.)

9.2.4.2.4 Tannins

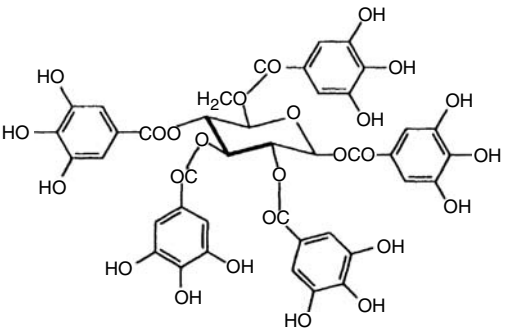
A rigorous definition of tannins does not exist, and many substances varying in structure are included under this name. Tannins are special phenolic compounds and are given this name simply by virtue of their ability to combine with proteins and other polymers such as polysaccharides, rather than their exact chemical nature. They are functionally defined, therefore, as water-soluble polyphenolic compounds with molecular weights between 500 and 3000 that have the ability to precipitate alkaloids, gelatin, and other proteins. They occur in the bark of oak trees and in fruits. The chemistry of tannins is complex. They are generally considered as two groups: (1) proanthocyanidins, also referred to as "condensed tannins" (previously discussed) and (2) glucose polyesters of gallic acid of hexahydroxydiphenic acids (Figure 9.33). The latter group is also known as hydrolyzable tannins, because they consist of a glucose molecule bonded to several phenolic moieties. The most important example is glucose bonded to gallic acid and the lactone of its dimer, ellagic acid. Tannins range in color from yellowish-white to light brown and contribute to astringency in foods. They contribute to the color of black teas when catechins are converted to theaflavins and thearubigins during fermentation. Their ability to precipitate proteins makes them valuable as clarifying agents.

9.2.4.3 Quinoids and Xanthones

Quinones are phenolic compounds varying in molecular weight from a monomer, such as 1,4-benzoquinone, to a dimer, 1,4-naphthaquinone, to a trimer, 9,10-anthraquinone, and finally to a polymer represented by hypericin (Figure 9.34). They are widely distributed in plants, specifically trees, where they contribute to the color of wood. Most quinones are bitter in taste. Their contribution

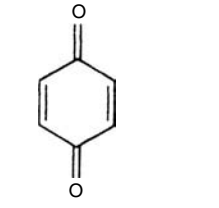


Proanthocyanidin

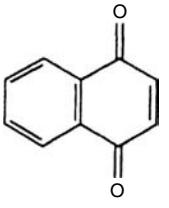


Pentagalloyl glucose

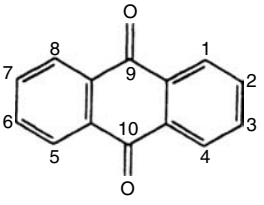
FIGURE 9.33 Structure of tannins.



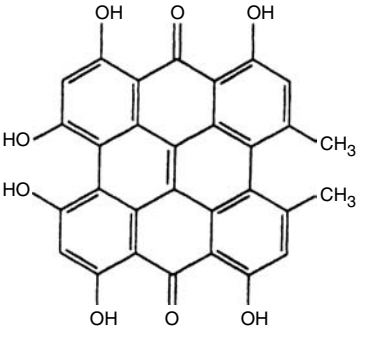
1,4-Benzoquinone



1,4-Napthoquinone



9,10-Anthraquinone



Hypericin

FIGURE 9.34 Structure of quinones.

to color of plants is minimal. They contribute to some of the darker colors, to the yellows, oranges, and browns of certain fungi and lichens, and to the reds, blues, and purples of sea lilies and coccid insects. Compounds with complex substitutents such as naphthoquinone and anthraquinones occur in plants, and these have deep purple to black hues. Further color changes can occur *in vitro* under alkaline conditions by the addition of hydroxyl groups. Xanthone pigments are yellow, phenolic pigments and they are often confused with quinones and flavones because of their structural characteristics. The xanthone mangiferin (Figure 9.35) occurs as a glucoside in mangoes. They are easily distinguishable from quinones by their spectral characteristics.

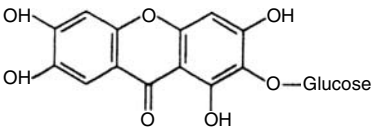


FIGURE 9.35 Structure of mangiferin.

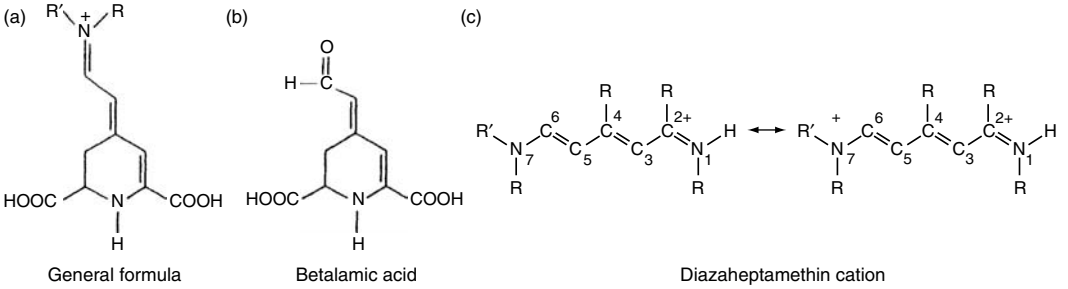


FIGURE 9.36 General formulas of betalains.

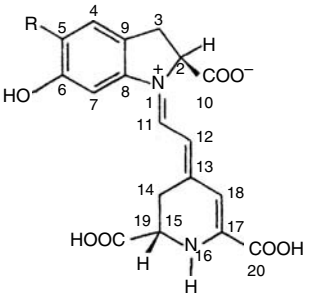
9.2.5 Betalains

9.2.5.1 Structure

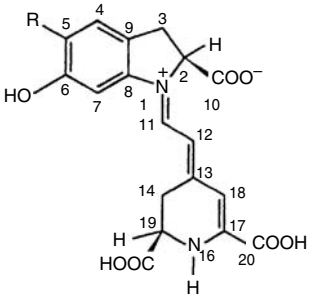
Plants containing betalains have colors similar to plants containing anthocyanins. Betalains are a group of pigments containing betacyanins (red) and betaxanthins (yellow) and their color is not affected by pH, contrary to the behavior of anthocyanins. They are water soluble and exist as internal salts (zwitterions) in the vacuoles of plant cells. Plants containing these pigments are restricted to ten families of the order Centrospermae. The presence of betalains in plants is mutually exclusive of the occurrence of anthocyanins. The general formula for betalains (Figure 9.36a) represents condensation of a primary or secondary amine with betalamic acid (BA) (Figure 9.36b). All betalain pigments can be described as a 1,2,4,7,7-pentasubstituted 1,7-diazaheptamethin system (Figure 9.36c). When R′ does not extend conjugation of the 1,7-diazaheptamethine system, the compound exhibits maximum light absorption at about 480 nm, characteristic of yellow betaxanthins. If the conjugation is extended at R′ the maximum light absorption shifts to approximately 540 nm, characteristic of red betacyanins.

Betacyanins are optically active because of the two chiral carbons C-2 and C-15 (Figure 9.37). Hydrolysis of betacyanin leads to either betanidin (Figure 9.37), or the C-15 epimer isobetanidin (Figure 9.37d), or a mixture of the two isomeric aglycones. These aglycones are shared by all betacyanins. Differences between betacyanins are found in their glucoside residue. Common vegetables containing betalains are red beet and amaranth. The latter is either consumer fresh as "greens" or at the mature state as grain. The most studied betalains are those of the red beet. The major betacyanins in the red beet are betanin and isobetanin (Figure 9.37b and 9.37e), while in amaranth they are amaranthin and isoamaranthin (Figure 9.37c and 9.37f).

The first betaxanthin isolated and characterized was indicaxanthin (Figure 9.38a). Structurally these pigments are very similar to betacyanins. Betaxanthins differ from betacyanins in that the indole nucleus is replaced with an amino acid. In the case of indicaxanthin the amino acid is proline. Two betaxanthins have been isolated from beet, vulgaxanthin I and II (Figure 9.38b). They differ from indicaxanthin in that the proline has been replaced by glutamine or glutamic acid, respectively. Although only a few betaxanthins have been characterized to date, considering the number of amino acids available, it is likely a large number of different betaxanthins exist.

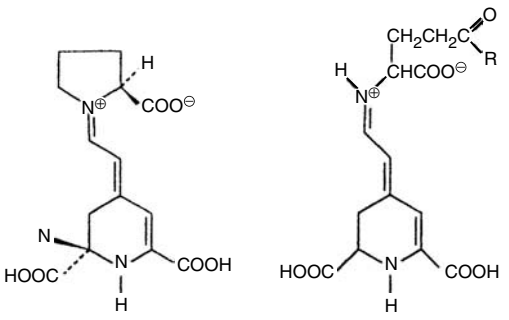


(a) Betanidin, R = –OH
(b) Betanin, R = –Glucose
(c) Amaranthin, R = 2′-glucuronic acid-glucose



(d) Isobetanidin, R = –OH
(e) Isobetanin, R = –Glucose
(f) Isoamaranthin, R = 2′-glucuronic acid-glucose

FIGURE 9.37 Structure of betacyanins.



(a) Indicaxanthin

(b) Vulgaxanthin-I, R = $-NH_2$
Vulgaxanthin-II, R = –OH

FIGURE 9.38 Structure of betaxanthins.

9.2.5.2 Physical Properties

Betalains absorb light strongly. The molar absorptivity value is 1120 for betanin and 750 for vulgaxanthin, suggesting high tinctorial strength in the pure state. The spectra of betanin solutions at pH values between 4.0 and 7.0 do not change, and they exhibit maximum light absorbance at 537–538 nm. No change in hue occurs between these pH values. Below pH 4.0, the absorption maximum shifts toward a slightly shorter wavelength (535 nm at pH 2.0). Above pH 7.0, the absorption maximum shifts toward a longer wavelength (544 nm at pH 9.0). The chemical characteristics, biosynthesis, and the stability properties of these pigments as food colorants have been reviewed [29].

9.2.5.3 Chemical Properties

Like other natural pigments, betalains are affected by several environmental factors.

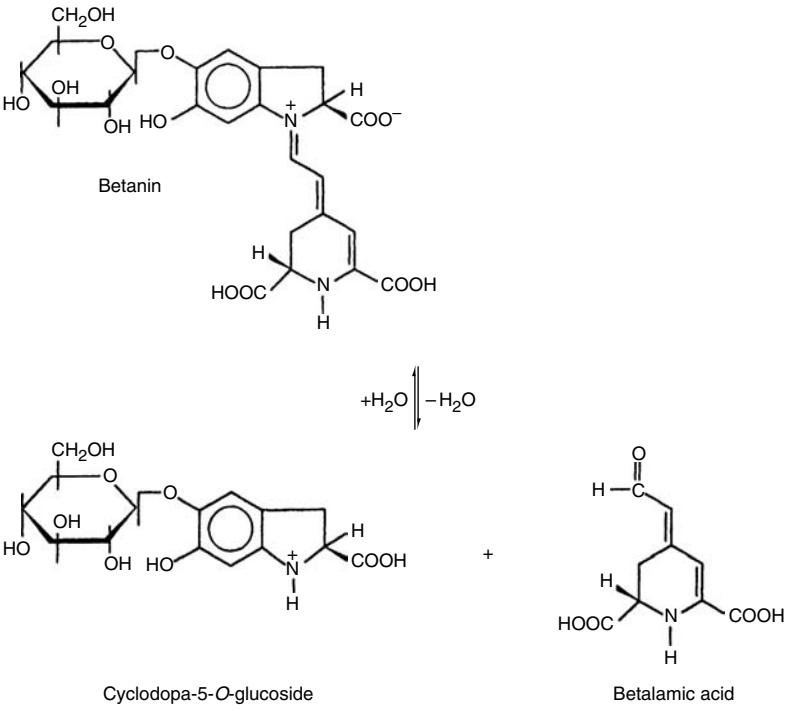


FIGURE 9.39 Degradation reaction of betanin.

TABLE 9.9
Effect of Oxygen and pH on the Half-Life Values of Betanin in Aqueous Solution at 90°C

	Half-Life Values of Betanin (min)	
pH	**Nitrogen**	**Oxygen**
3.0	56 ± 6	11.3 ± 0.7
4.0	115 ± 10	23.3 ± 1.5
5.0	106 ± 8	22.6 ± 1.0
6.0	41 ± 4	12.6 ± 0.8
7.0	4.8 ± 0.8	3.6 ± 0.3

Source: Adapted from Fox, J. B., Jr. (1966). *J. Agric. Food Chem.* 14:207–210.

9.2.5.3.1 Heat and/or Acidity

Under mild alkaline conditions, betanin degrades to BA and cyclodopa-5-*O*-glucoside (CDG) (Figure 9.39). These two degradation products also form during heating of acidic betanin solutions or during thermal processing of products containing beet root, but more slowly. The reaction is pH dependent (Table 9.9), and the greatest stability is in the pH range of 4.0–5.0. It should be noted that the reaction requires water; thus when water is unavailable or limited, betanin is very stable. It follows that a decrease a_w will cause a decrease in the degradation rate of betanin [106]. An a_w of 0.12 (moisture content of about 2%; dry weight basis), is recommended for optimal storage stability

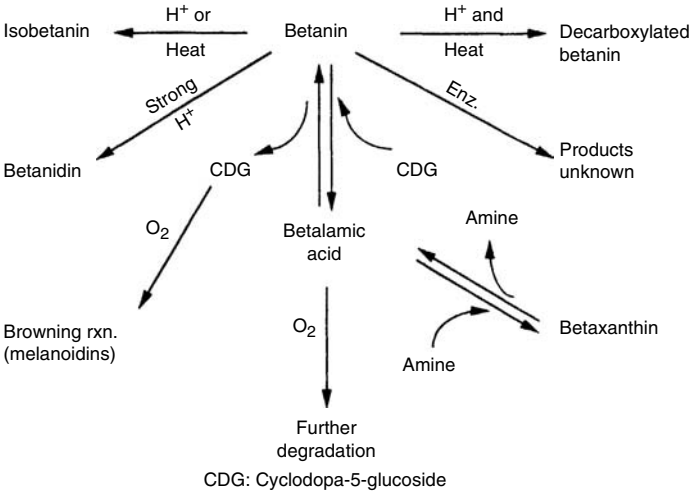


FIGURE 9.40 Degradation of betanin under acid and/or heat.

of the pigments in beet powder [24]. No studies have been done concerning the degradation mechanism of betaxanthins. Since both betacyanins and betaxanthins possess the same general structure, the mechanisms for degradation of betanin is likely to apply.

Degradation of betanin to BA and CDG is reversible, and therefore partial regeneration of the pigment occurs following heating. The mechanism proposed for regeneration involves a Schiff-base condensation of the aldehyde group of BA and the nucleophilic amine of CDG (Figure 9.39). Regeneration of betanin is maximized at an intermediate pH range (4.0–5.0) [43,61]. It is of interest that canners traditionally, for reasons not necessarily known to them, examine canned beets several hours after processing to evaluate color, thus taking advantage of regeneration of the pigment(s).

Betacyanins, because of the C-15 chiral center (Figure 9.37), exist in two epimeric forms. Epimerization is brought about by either acid or heat. It would therefore be expected that during heating of a food containing betanin, the ratio of isobetanin to betanin would increase. However, epimerization does not affect its color. It has also been shown that when betanin in aqueous solution is heated, decarboxylation can occur. Evidence for this evolution of carbon dioxide is loss of the chiral center. The rate of decarboxylation increases with increasing acidity [62]. Degradation reactions of betanin under acid and/or heat are summarized in Figure 9.40.

9.2.5.3.2 Oxygen and Light

Another major factor that contributes to degradation of betalains is the presence of oxygen. Oxygen in the head space of canned beets has long been known to accelerate pigment loss. In solutions containing a molar excess of oxygen over betanin, betanin loss follows apparent first-order kinetics. Betanin degradation deviates from first-order kinetics when the molar oxygen concentration is reduced to near that of betanin. In the absence of oxygen, stability is increased. Molecular oxygen has been implicated as the active agent in oxidative degradation of betanin. Because betalains are susceptible to oxidation, these compounds are also effective antioxidants [152]. Degradation of betanin in the presence of oxygen is influenced by pH (Table 9.9).

Oxidation of betalains accelerates in the presence of light. The presence of antioxidants, such as ascorbic acid and isoascorbic acid, improves their stability. Because copper and iron cations catalyze oxidation of ascorbic acid by molecular oxygen, they detract from the effectiveness of ascorbic acid as a protector of betalains. The presence of metal chelators (EDTA or citric acid) greatly improves the effectiveness of ascorbic acid as a stabilizer of betalains [3,9].

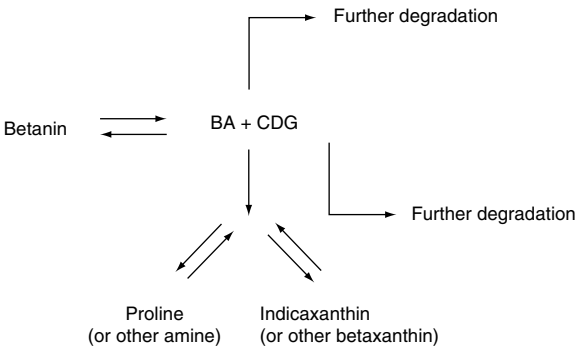


FIGURE 9.41 Formation of indicaxanthin from betanin in excess of proline.

Several phenolic antioxidants, including BHA, butylated hydroxytoluene, catechin, quercetin, nordihydroguaiartic acid, chlorogenic acid, and α-tocopherol, inhibit free-radical chain autoxidation. Since free-radical oxidation does not seem to be involved in betalain oxidation, these antioxidants are, not surprisingly, ineffective stabilizers of betanin. Similarly, sulfur-containing antioxidants such as sodium sulfite and sodium metabisulfite are not only ineffective stabilizers, they hasten loss of color. Sodium thiosulfite, a poor oxygen scavenger, has no effect on betanin stability. Thiopropionic acid and cysteine also are ineffective as stabilizers of betanin. These observations confirm that betanin does not degrade by a free-radical mechanism. The susceptibility of betalains to oxygen has limited their use as food colorants.

9.2.5.3.3 Conversion of Betacyanin to Betaxanthin

In 1965, it was shown that the betaxanthin indicaxanthin could be formed from betacyanin, betanin, and an excess of proline in the presence of 0.6 N ammonium hydroxide under vacuum. This was the first conclusive evidence of a structural relationship between betacyanin and betaxanthin. It was further demonstrated that formation of betaxanthin from betanin involved condensation of the betanin hydrolysis product BA, and an amino acid (Figure 9.41) [59,112,113].

Information on the stability of betaxanthins is limited. Similar to betacyanins, their stability is pH dependent. Shown in Figure 9.42 are the heat stability differences between the betacyanin, betanin, and the betaxanthin, vulgaxanthin, under similar experimental conditions. The mechanism in Figure 9.41 suggests that an excess of the appropriate amino acid will shift the equilibrium toward the corresponding betaxanthin and will reduce the quantity of BA in solution. An excess of an amino acid increases the stability of the betaxanthin formed by reducing the amount of BA available for degradation. This effect is illustrated in the two upper curves of Figure 9.42. Conversion of betacyanin to betaxanthin can occur in protein-rich foods, and this accounts for the loss of red color in these types of foods that are colored with betalains.

9.3 FOOD COLORANTS

9.3.1 Regulatory Aspects

9.3.1.1 United States

In the United States, colorant use is controlled by the 1960 Color Additive Amendment to the U.S. Food, Drug and Cosmetic Act of 1938. The amendment classifies colorants into two categories: certified colors and colors exempt from certification. Certified colors are synthetic dyes and they are not found in nature. Certification means that the dye meets specific government quality standards. Samples of each production batch must be submitted to an FDA laboratory for determination of

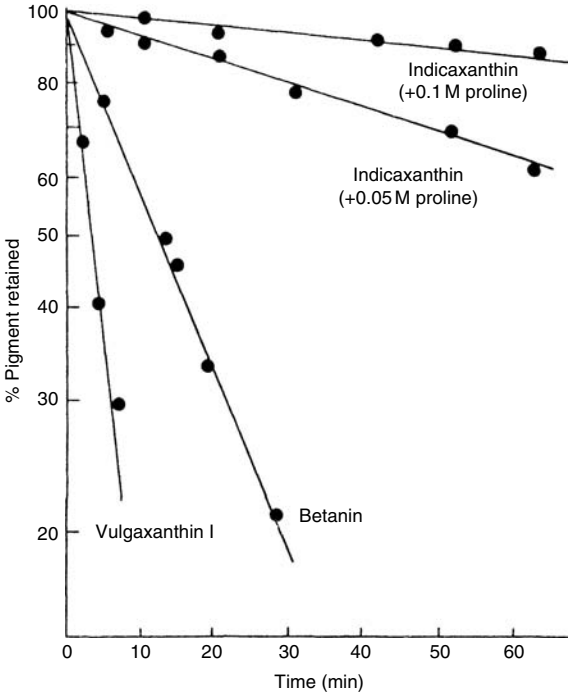


FIGURE 9.42 Stability comparison of betanin vulgaxanthin I and of indicaxanthin in presence of proline in solution at pH 5.0, 90°C, under atmospheric conditions.

compliance. If the batch is in compliance it is assigned an official lot number. Certified dyes are further classified as either permanently or provisionally listed. A "provisionally" approved certified dye can be legally used pending completion of all scientific investigation needed for determination for or against permanent approval. The same considerations apply to lakes.

Colorants exempt from certification are natural pigments or specific synthetic dyes that are nature identical. An example of the latter is β-carotene that is widely distributed in nature but also can be synthesized to achieve a "nature identical" substance.

The Color Additive Amendment includes a simplified nomenclature for certified dyes. Rather than the use of long and difficult common names, certified dyes are referred to by a number and the abbreviation FD&C, D&C, or Ext. (external) D&C. FD&C stands for FD&Cs, and these colorants may be used in either foods, drugs, or cosmetics. D&C and Ext. (external) D&C dyes can be used only in drugs or cosmetics. Thus, the certified dye sunset yellow FC has the designation FD&C Yellow No. 6. The current list of permitted certified dyes contains seven colorants for general use (Table 9.10). Two additional dyes, Orange B and Citrus Red No. 2, may be used; however, their use is restricted to specific applications. Orange B may be used only for coloring the casings or surfaces of frankfurters and sausages, and its use in these applications is restricted to no more than 150 ppm by weight of the finished product. Citrus Red No. 2 may be used only for coloring the skins of oranges not intended or used for processing, and its use in this application is restricted to no more than 2 ppm based on the whole fruit weight.

Adoption of the Nutritional Labeling and Education Act of 1990 that became effective in 1994 makes mandatory the individual listing of certified colors, by their abbreviated names. Colors exempt from certification must be declared, but they can be listed generically as "artificial color" or any other specific or generic name for the colorant. However, the use of the term "natural" referred to colorants is prohibited since it may lead the consumer to believe that the color is derived from the food itself. Color additives currently exempt form certification are listed in Table 9.11.

TABLE 9.10
Certified Color Additives Currently Permitted for General Use[a] and Their Corresponding Nomenclature According to the European Economic Community

	Status			
Name	**Dye**	**Lake**	**Common Name**	**E-Numbers[b]**
FD&C Blue No. 1	Permanent	Provisional	Brilliant blue	E133
FD&C Blue No. 2	Permanent	Provisional	Indigotine	E132
FD&C Green No. 3	Permanent	[c]	Fast green	[d]
FD&C Red No. 3	Permanent	Provisional	Erythrosine	E123
FD&C Red No. 40	Permanent	Permanent	Allura red	E129
FD&C Yellow No. 5	Permanent	Provisional	Tartrazine	E102
FD&C Yellow No. 6	Permanent	Provisional	Sunset yellow	E110

[a] E-numbers: Numbers listed in the European Economic Community.
[b] Use of lake of FD&C Red No. 3 was terminated effective January 29, 1990.
[c] Not listed.

Source: Code of Federal Regulations, Title 21, Chapter 1, Part 73, revised as of April 2004.

9.3.1.2 International

Colors are added to foods in many countries of the world, but the type of colorants permitted for use varies greatly among countries. Since international trade is becoming increasingly important, color legislation is now of international concern. Unfortunately, a worldwide list of permitted color additives does not exist; therefore, color additives have, in some instance, become trade barriers for foods. In the United States, for example, FD&C Red No. 40 is permitted for food use, whereas FD&C Red No. 2, since 1976, is no longer permitted. At the other extreme, Norway prohibits the use of any synthetic dye in the manufacturing of foods. Legislative authorities of the EEC have attempted to achieve uniform color additive legislation for Common Market Countries. Each permitted color additive has been assigned an E-number (E = Europe). Listed in Table 9.10 are the E-numbers for some of the artifical dyes approved in the EEC and their equivalent FD&C number. Similar information for EEC natural colorants is given in Table 9.11. In reviewing these tables, it must be remembered that use of a colorant may be restricted to one or more specific products. An EEC general-use colorant also may not be approved by every country in the EEC. In general, it is apparent that greater latitude of use of both synthetic and natural colorants is currently allowed among EEC countries than in the United States and Canada. Historically, Japan had a very strict policy on the use of colorants in foods and synthetic dyes were banned. However, Japan has recently expanded its list of designated additives that includes the food coloring agents. As of December 2004, the list includes not only the nonchemically synthesized food additives, but also a list of 12 different synthetic dyes and many corresponding lakes, all with restricted uses. Among the 7 FD&C dyes and lakes approved by the FDA, all but FD&C Yellow No. 6 are currently included in the Japanese list of designated additives under the food sanitation law [70]. The reader is referred to the Food Additives Tables (Elsevier, Amsterdam) for further details.

The FAO and the WHO have attempted to harmonize food regulations among countries through their Codex Alimentarius. FAO and WHO formed the Joint WHO/FAO Expert Committee on Food Additives (JEFCA) to work on a global basis to asses the safety of food additives. The JEFCA has devised "acceptable daily intakes" (ADI) for food additives, including colorants (Table 9.12). Worldwide efforts toward establishing safety of colorants should lead to internationally accepted regulations for colorant use in foods.

TABLE 9.11
U.S. Color Additives Currently Exempt from Certification, Color Use Limitation and Their Corresponding Nomenclature According to the European Economic Community

Section	Color Additive	U.S. Food Use Limit	E-Numbers[a]
73.30	Annatto extract	GMP	E160b
73.35	Astaxanthin	<80 mg/kg fish feed	
73.40	Dehydrated beets (beet powder)	GMP	E162
73.50	Ultramarine blue	Salt for animal feed	NL
73.75	Canthaxanthin	<30 mg/lb of solid/semisolid food or pint of liquid <4.41 mg/kg chicken feed	E161g
73.85	Caramel	GMP	E150
73.90	[β]-Apo-8′-carotenal	<15 mg/lb solid/semisold food or 15 mg/pint liquid food	E160a
73.95	[β]-Carotene	GMP	E150
73.100	Cochineal extract; carmine	GMP	E120
73.125	Sodium copper chlorophyllin	<0.2% for dry mix citrus based beverages	E141
73.140	Toasted partially defatted cooked cottonseed flour	GMP	NL
73.160	Ferrous gluconate	GMP for ripe olives only	NL
73.165	Ferrous lactate	GMP for ripe olives	
73.169	Grape color extract	GMP for nonbeverage foods	E163
73.170	Grape skin extract (enocianina)	GMP for beverages	E163
73.185	Haematococcus algae meal	<80 mg/kg salmonid fish feed	
73.200	Synthetic iron oxide	Pet food up to 0.25%	E172
73.250	Fruit juice	GMP	NL
73.260	Vegetable juice	GMP	NL
73.275	Dried algae meal	GMP for chicken food	NL
73.295	Tagetes (Aztec marigold) meal and extract	GMP	NL
73.300	Carrot oil	GMP	NL
73.315	Corn endosperm oil	GMP for chicken feed	NL
73.340	Paprika	GMP	E160c
73.345	Paprika oleoresin	GMP	E160c
73.355	Phaffia yeast	<80 mg/kg salmonid fish feed	
73.450	Riboflavin	GMP	E101
73.500	Saffron	GMP	NL
73.575	Titanium dioxide	<1% by weight of food	E171
73.600	Turmeric	GMP	E100
73.615	Turmeric oleoresin	GMP	E100

GMP = Good Manufacturing Practices.

[a] E-numbers: Numbers listed in the European Economic Community. In addition, the EEC allows the use of anthocyanins/juice concentrates (E163), beet pigments (E162), and chlorophylls (E140).

Source: From Code of Federal Regulations, Title 21, Chapter 1, Part 73, revised as of April 2004.

9.3.2 Properties of Certified Dyes

The safety of certified colors has received much public attention in recent years. The root of the concern can in part be attributed to the unfortunate association of synthetic colors to the original term "coal-tar" dyes. The public's concept of coal tar is a thick black substance unsuitable for use in

TABLE 9.12
Acceptable Daily Intake of Some Synthetic and Natural Colorants[a]

Pigment	E-Number	ADI (JEFCA) (mg/kg body weight)
Curcumin	E100	0.1
Riboflavin[b]	E101	0.5
Tartrazine	E102	7.5
Carmines	E120	5.0
Erythrosine	E127	0.1
Brilliant blue FCF	E133	12.5
Chlorophylls[b]	E140	NS
Caramel	E150	200
β-Carotene	E160a	5.0
Annatto	E160b	0.065
Paprika	E160c	NS
Beetroot red	E162	NS
Anthocyanins	E163	NS
Grape skin extract[b]	E163	2.5

[a] Modified from Henry, 2000. In Natural Food Colorants, IFT Symposium Series.
[b] From Francis, F. J. (1999). *Colorants*, Eagan Press Handbook Series, St Paul, Minnesota, USA.

NS: not specified.

foods. The fact is that raw materials for synthesis of colors are highly purified before use. The final product is a specific chemical that bears little relationship to the term coal tar.

Certified dyes fall into four basic chemical classes: azo, triphenylmethane, xanthine, or indigo type dyes. Listed in Table 9.13 are the FD&C dyes, their chemical class, and some of their properties. The structures are shown in Figure 9.43. Listed in Table 9.14 are solubility and stability data for EEC dyes.

A simplified sequence for chemical synthesis of FD&C Green No. 3, a triphenylmethane dye, is given in Figure 9.44. In manufacturing any dye the major difficulty is to meet the specifications of purity given for certification in the United States (Code of Federal Regulations, Title 21, Part 70–83). The color manufacturing industry not only meets these purity specifications, but also most manufacturers exceed them.

The pure dye content of a typical certified colorant is 86–96%. Variation of 2–3% in total dye content of a basic color is of little practical significance since such variation has no significant effect on the ultimate color of a product. The moisture content of dye powders is between 4 and 5%. The salt (ash) content of the dye powder is approximately 5%. The high ash content comes from the salt used to crystallize (salt out) the colorant. Although it is technically possible to remove the sodium chloride used, such steps would be costly and would have minimal benefit.

All water-soluble FD&C azo dyes are acidic, and their physical properties are quite similar. Chemically they are reduced easily by strong reducing agents and therefore are susceptible to oxidizing agents. FD&C triphenylmethane dyes (FD&C Green No. 3 and FD&C Blue No. 1) are similar in structure, differing only in one —OH group. Differences in solubility and stability are therefore minor. Substitution of a sulfonic acid group for a hydroxyl group in either of these dyes improves stability to light and resistance to alkali. Alkali decolorization of a triphenylmethane dye involves formation of a colorless carbinol base (Figure 9.45). The ortho-substituted sulfonic acid group sterically hinders access of the hydroxyl ion to the central carbon atom, thus preventing formation of the carbinol base.

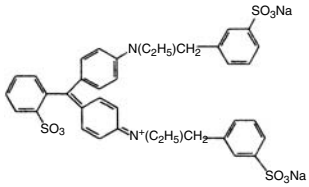
FD&C Red No. 3 is the only xanthine type-dye. The structure of FD&C Red No. 3 suggests that the dye is insoluble in acids, quite stable to alkali, and exhibits strong fluorescence. The water insoluble

TABLE 9.13
Certified Colorants and Their Chemical and Physical Properties

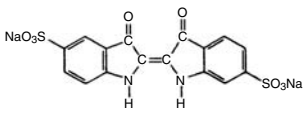
Common Name and FD&C Number	Dye type	Solubility (g/100 ml)[a]								Stability[b]									
		Water		Propolyne Glycol		Alcohol		Glycerine		At pH:									
		25°C	60°C	25°C	60°C	25°C	60°C	25°C	60°C	3.0	5.0	7.0	8.0	Light	10% AcOH	10% NaOH	250 ppm SO_2	1% Ascorbic Acid	1% Sodium Benzoate
FDC Blue No. 1	Triphenylmethane	20.0	20.0	20.0	20.0	0.35	0.20	20.0	20.0	4	5	5	5	3	5	4	5	4	6
FDC Blue No. 2	Indigo	1.6	2.2	0.1	0.1	In	0.007	20.0	20.0	3	3	2	1	1	1	2[b2]	1	2	4
FDC Green No. 3	Triphenylmethane	20.0	20.0	20.0	20.0	0.01	0.03	1.0	1.3	4	4	4	4[b1]	3	5	2[b1]	5	4	6
FDC Red No. 3	Xanthine	9.0	17.0	20.0	20.0	In	0.01	20.0	20.0	In	In	6	6	2	In	2	In	In	5
FDC Red No. 40	Azo	22.0	26.0	1.5	1.7	0.001	0.113	3.0	8.0	6	6	6	6	5	5	3[b1]	6	6	6
FDC Yellow No. 5	Azo	20.0	20.0	7.0	7.0	In	0.201	18.0	18.0	6	6	6	6	5	5	4	3	3	6
FDC Yellow No. 6	Azo	19.0	20.0	2.2	2.2	In	0.001	20.0	20.0	6	6	6	6	3	5	5	3	2	6

[a] In, Insoluble.

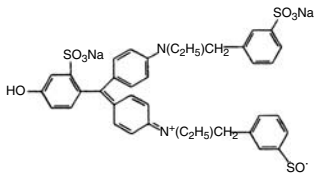
[b] 1 = fades; 2 = considerable fade; 3 = appreciable fade; 4 = slight fade; 5 = very slight fade; 6 = no change; b1 = hue turns blue; b2 = hue turns yellow.



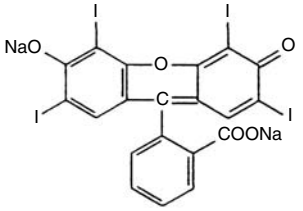
FD&C Blue No. 1



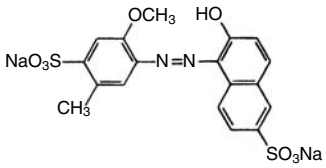
FD&C Blue No. 2



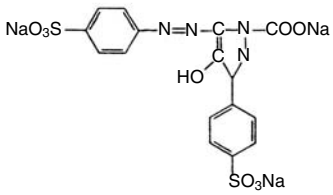
FD&C Green No. 3



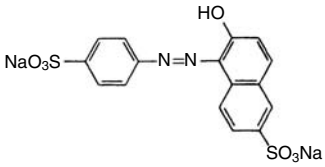
FD&C Red No. 3



FD&C Red No. 40



FD&C Yellow No. 5



FD&C Yellow No. 6

FIGURE 9.43 Structures of certified color additives currently permitted for general use in the United States.

TABLE 9.14
Chemical and Physical Properties of Common EEC Dyes

	Solubility (g/100 mL) at 16°C				Stability[a]				
								pH	
Name and EEC-Number	Water	Propylene Glycol	Alcohol	Glycerine	Light	Heat	SO_2	3.5/4.0	8.0/9.0
Quinoline yellow, E104	14	<0.1	<0.1	<0.1	6	5	4	5	2
Ponceau 4R, E124	30	4	<0.1	0.5	4	5	3	4	1
Carmoisine, E122	8	1	<0.1	2.5	5	5	4	4	3
Amaranth, E123	5	0.4	<0.1	1.5	5	5	3	4	3
Patent blue, E131	6	2	<0.1	3.5	7	5	3	1	2
Green S, E142	5	2	0.2	1.5	3	5	4	4	3
Chocolate brown HT, E156	20	15	insoluble	5	5	5	3	4	4
Brilliant black BN, E151	5	1	<0.1	<0.5	6	1	1	3	4

[a] 1 = fades; 2 = considerable fade; 3 = appreciable fade; 4 = slight fade; 5 = very slight fade; 6 = no change.

form (lake) of FD&C Red No. 3 is no longer permitted for use in foods because of toxicologic concerns. Although the dye in its water soluble form is permanently listed, its long term future is questionable. FD&C Blue No. 2 is the only indigoid-type dye. It is made from indigo, one of the oldest known and most extensively utilized natural pigments. The pigment is derived from various species of the indigo plant found in India. Until its synthesis, extracted dye was a major product of commerce. FD&C Blue No. 2 is made by sulfonating indigo, yielding 5,5′-indigotin disulfonate (Figure 9.46). The color is a deep blue, compared to the greenish-blue of FD&C Blue No. 1. The dye

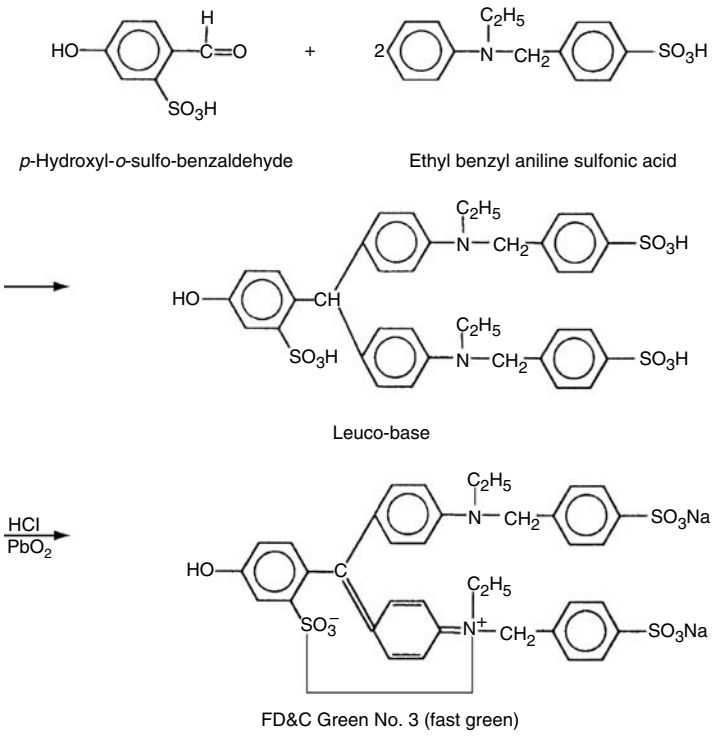


FIGURE 9.44 Synthesis of FD&C Green No. 3 (fast green).

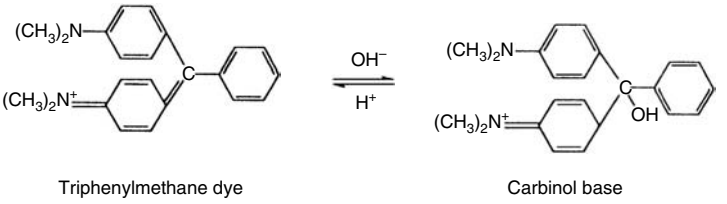


FIGURE 9.45 Formation of a colorless carbinol base from a triphenylmethane dye.

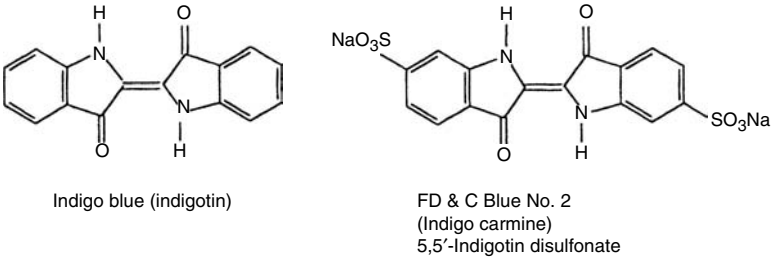


FIGURE 9.46 Structures of indigoid-type dyes.

has the lowest water solubility and poorest light resistance of any of the FD&C dyes, but is relatively resistant to reducing agents.

In general, conditions most likely to cause discoloration or precipitation of certified dyes are the presence of reducing agents or heavy metals, exposure to light, excessive heat, or exposure to acid or

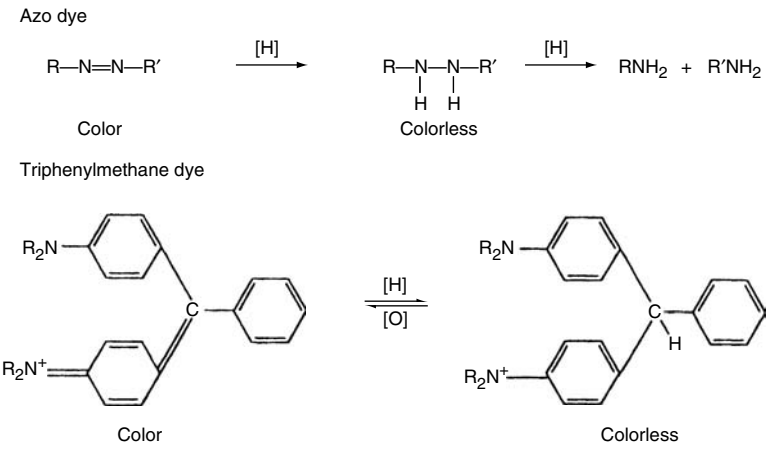


FIGURE 9.47 Reduction of azo or triphenylmethane dyes to colorless products.

alkali. Many of the conditions causing failure of the dyes can be prevented in foods. Reducing agents are most troublesome. Reduction of the chromophores of azo and triphenylmethane dyes is shown in Figure 9.47. Azo dyes are reduced to the colorless hydrazo form or sometimes to the primary amine. Triphenylmethane dyes are reduced to the colorless leuco base. Common reducing agents in foods are monosaccharides (glucose, fructose), aldehydes, ketones, and ascorbic acid.

Free metals can combine chemically with many dyes causing loss of color. Of most concern are iron and copper. The presence of calcium and magnesium can result in the formation of insoluble salts and precipitates.

9.3.3 Use of Certified Colors

There are some practical advantages of the use of synthetic dyes. In general, they have high tinctorial power, so only small amounts are required to provide the desired color, resulting also in low cost. In addition, they have very high stability to processing and storage conditions as compared to the "natural" counterparts. Also, they are available in the water soluble (dye) and water insoluble (lake) forms. Better uniformity in incorporating a water-soluble dye in foods is achieved if the dye is first dissolved in water. Distilled water should be used to prevent precipitation. Liquid colors of various strengths can be purchased from manufacturers. Dye concentration in these preparations usually does not exceed 3% to avoid overcoloring. Citric acid and sodium benzoate are commonly added to liquid preparations to prevent microbial spoilage.

Many foods contain low levels of moisture making it impossible to completely dissolve and uniformly distribute a dye. The result is a weak color and/or a speckled effect. This is a potential problem in hard candy that has a moisture content of $<1\%$. The problem is averted by using solvents other than water, such as glycerol or propylene glycol (Table 9.13). A second approach to overcoming problems of poor dispersion of dyes in foods with low moisture contents is the use of "lakes." Lakes exist as dispersions in food rather than in solution. They range in dye content from 1 to 40%. A large dye content does not necessarily lead to intense color. Particle size is of key importance—the smaller the particle size, the finer will be the dispersion and the more intense will be the color. Special grinding techniques used by color manufacturers have made it possible to prepare lakes with a mean particle size of less than 1 μm.

As with dyes, predispersion of lakes in glycerol, propylene glycol, or edible oils is often required. Predispersion helps prevent agglomeration of particles, and thereby helps develop full color intensity and reduces the incidence of speckled products. Lake dispersions vary in dye content from 15 to

35%. A typical lake dispersion may contain 20% FD&C lake A, 20% FD&C lake B, 30% glycerol, and 30% propylene glycol, resulting in a final dye content of 16%.

Color manufacturers also prepare color pastes or solid cubes of dyes or lakes. A paste is made with the addition, for example, of glycerol as a solvent and powdered sugar to increase viscosity. Colorants, in the form of cubes, are achieved by adding gums and emulsifiers to color dispersions in the manufacturing process.

9.3.4 Colors Exempt from Certification

A brief description of each of the colorants listed in Table 9.11 follows.

Annatto extract is the extract prepared from Annatto seed, *Bixa orellana* L. Several food-grade solvents can be used for extraction. Supercritical carbon dioxide extraction has been tested as an alternative to using standard organic solvents. This technology, however, has not yet been commercialized. The main pigment in annatto extract is the carotenoid bixin. Upon saponification of bixin, the methyl ester group is hydrolyzed and the resulting diacid is called norbixin (Figure 9.48). Bixin and norbixin differ in solubility and form the basis for oil- and water-soluble annatto colors, respectively.

Dehydrated beet is obtained by dehydrating the juice of edible whole beets. The pigments in beet colorants are betalains (betacyanins [red] and betaxanthins [yellow]). The ratio of betacyanin/betaxanthin will vary depending on the cultivar and maturity of beets. Beet colorant can also be produced under the category of "vegetable juice." This type of beet colorant is obtained by concentrating beet juice under vacuum to a solid content sufficient to prevent spoilage (about 60% solids).

Canthaxanthin (β-carotene-4,4′-dione), β-apo-8′-carotenal, and β-carotene are synthesized carotenoids and are regarded as "nature identical." Structures of these compounds are shown in Figure 9.49.

Cochineal extract is the concentrate produced from an aqueous-alcoholic extract of the cochineal insect, *Dactylopius coccus* L. Costa. The coloring is principally due to carminic acid, a red pigment (Figure 9.50). The extract contains about 2–3% carminic acid. Colorants with carminic acid concentrations of up to 50% are also produced. These colorants are sold under the name carmine colors.

Sodium copper chlorophyllin is a green to black powder prepared from chlorophyll by saponification and replacement of magnesium by copper. Chlorophyll is extracted from alfalfa using acetone, ethanol, and/or hexane. It may be safely used to color citrus-based dry beverage mixes in an amount not exceeding 0.2% in the dry mix.

Caramel is a dark-brown liquid produced by heat-induced caramelization of carbohydrates.

Toasted, partially defatted, cooked cottonseed flour is prepared as follows: cottonseed is delineated and decorticated; the meats are screened, aspirated, and rolled; moisture is adjusted; the meats are heated and the oil is expressed; and the cooked meats are cooled, ground, and reheated to obtain a product varying in shade from light brown to dark brown.

Ferrous gluconate is a yellowish-gray powder with a slight odor resembling that of burnt sugar.

Ferrous lactate is a greenish-white powder used for the coloring of ripe olives.

Grape skin extract is a purplish-red liquid prepared from an aqueous extract of pomace remaining after grapes have been pressed to remove the juice. The coloring matter of the extract consists mainly of anthocyanins. It is sold under the name "enocianina" and is restricted for coloring noncarbonated and carbonated drinks, beverage bases, and alcoholic beverages.

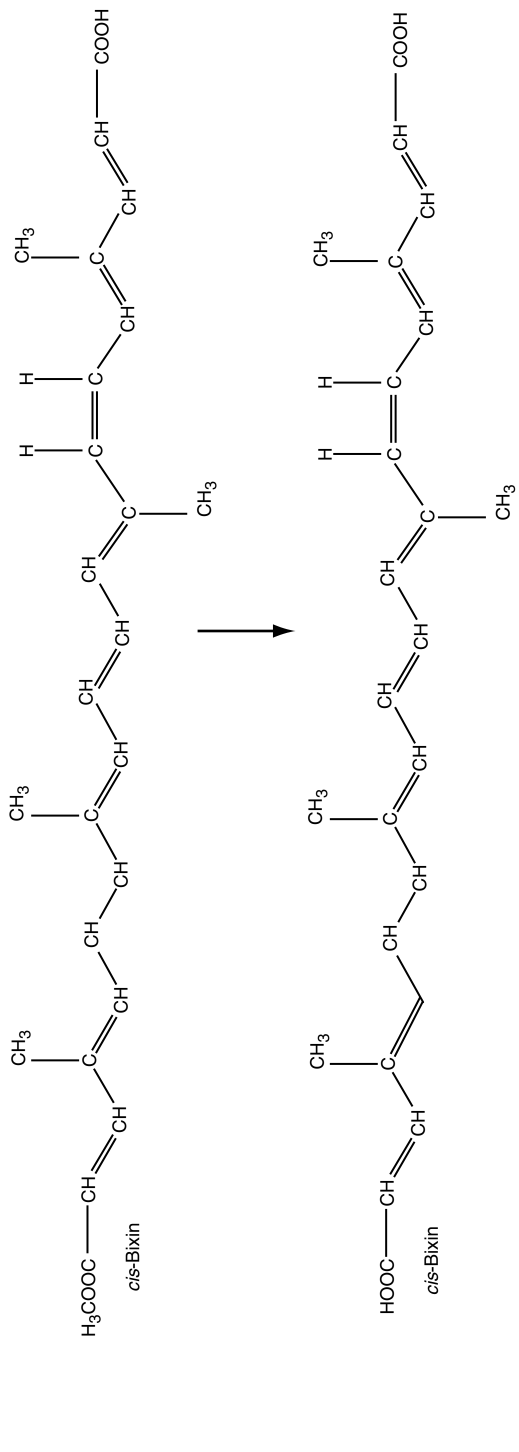
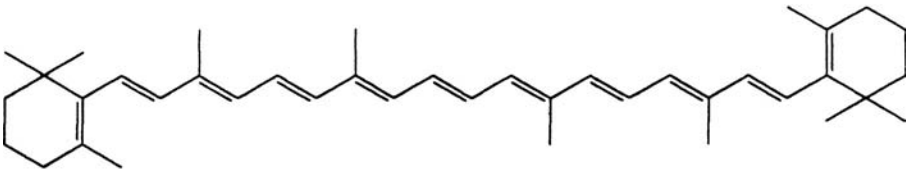
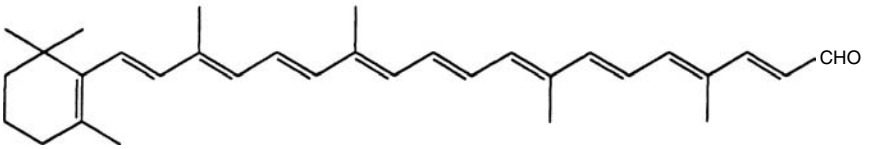


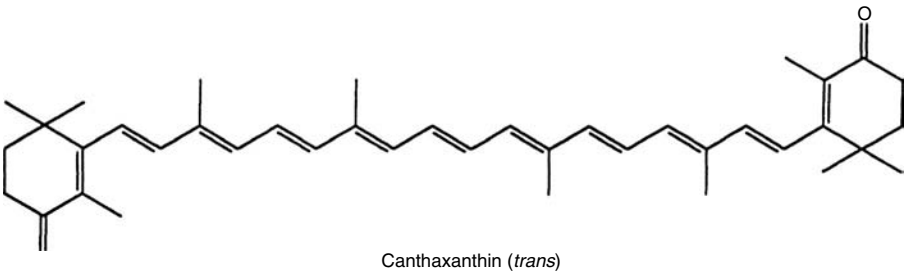
FIGURE 9.48 Formation of norbixin from bixin.



β-Carotene (*trans*)



β-Apo-8′-carotenal (C_{30}) (*trans*)



Canthaxanthin (*trans*)

FIGURE 9.49 Structure of "nature identical" carotenoids.

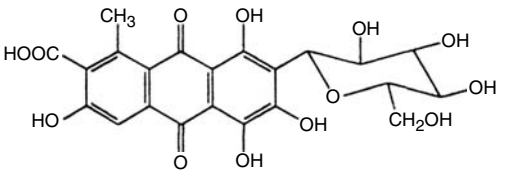


FIGURE 9.50 Structure of carminic acid.

Grape color extract is an aqueous solution of anthocyanin pigments made from Concord grapes or a dehydrated, water-soluble powder prepared from the aqueous solution. Grape color extract may be used for the coloring of nonbeverage foods as long as it does not affect their standard of identity.

Fruit and vegetable juices are acceptable color additives, and they can be used at single strength or as concentrated liquids. Depending on the source of the juice, pigments from many of the previously described classes can be involved. Beet and grape juice concentrates have been produced and marketed as colorants in this category. Grape juice concentrate, in contrast to grape skin extract, may be used in nonbeverage foods.

Carrot oil is produced by extracting edible carrots with hexane. The hexane is subsequently removed by vacuum distillation. The colorant is mainly α- and β-carotene and other minor carotenoids found in carrots.

Paprika or paprika oleoresin is either the ground dried pods of paprika (*Capsicum annum* L.) or an extract of this plant. In the production of oleoresin, several solvents may be used. The main colorant in paprika is capxanthin, a carotenoid.

Riboflavin or vitamin B_2 is an orange-yellow powder and is the native pigment in milk.

Saffron is the dried stigma of *Crocus sativul* L. Its yellow color is attributable to croxin, the digentiobioside of crocetin.

Titanium oxide is the whitest pigment known to date. It often contains silicon dioxide and/or aluminum oxide to aid dispersion in foods. These diluents may not exceed 2% of the total.

Turmeric and turmeric oleoresin are the grinded rhizomes or an extract of turmeric (*Curcuma longa* L.). The coloring matter in turmeric is curcumin. Several organic solvents may be used in the production of turmeric oleoresin.

Other colorants exempt from certification (Astaxanthin, Ultramarine blue, Haematococcus algae, iron oxide, dried algae meal [dried algae cells of the genus *Spongiococcum*], tagetes meal [dried ground flower petals of the Aztic marigold, *Tagetes erecta* L.], corn endosperm oil, and *Phaffia* yeast) are of little interest here since these colorants are restricted to use in animal feeds. They can, however, indirectly affect the color of foods.

The labeling declaration of added colors that are exempt from certification is somewhat controversial. Although colors exempt from certification are obtained from natural sources or are nature identical, they must be listed as "artificial color added." This is required because, in the vast majority of uses, the colorant added is not natural to the food product. Similar to certified colors, colors exempt from certification must be declared when used in foods in the United States.

9.3.5 Use of Colors Exempt from Certification

With the exception of synthetic, nature-identical pigments, exempt-from-certification colorants are chemically crude preparations. They are either totally unpurified materials or crude plant or animal extracts. Because of their impurity, relatively large amounts are needed to achieve the desired color. This has caused some to suggest that these pigments lack tinctorial strength and contribute undesirable flavors to a product. Neither criticism is necessarily true. Many pure natural pigments have high tinctorial strengths. This can be illustrated by comparing the absorptivity values of a 1% solution of a natural pigment with that of a synthetic dye. At wavelengths of maximum light absorption, the $A_{1cm}^{1\%}$ values for FD&C Red No. 40 and Yellow No. 6 are 586 and 569, respectively, whereas the $A_{1cm}^{1\%}$ for betanin, the main pigment component in beet powder, and β-carotene are 1120 and 2400, respectively. Furthermore, most pure pigments do not contribute to product flavor. The lack of tinctorial strength and the possible contribution to flavor by the unpurified natural colorants can be overcome by applying available technologies of separation and purification. Unfortunately, some of these advances in technology have not been sanctioned.

The demand for healthier and more wholesome foods has also resulted in an increased demand of colorants from natural sources. Health benefits associated with many naturally occurring pigments make them attractive alternatives to the use of synthetic colors. This, combined with legislative action and consumer awareness can lead to an increased interest in the use of colors from natural sources.

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10 Flavors

Robert C. Lindsay

CONTENTS

10.1 INTRODUCTION

10.1.1 General Philosophy

Knowledge of the chemistry of flavors is commonly perceived as a relatively recent development in food chemistry that evolved since the late 1950s with the advent of gas chromatography and fast-scan mass spectrometry. Although the availability of these instrumental tools has provided the means to definitively investigate the entire range of flavor substances, classic chemical techniques were elegantly applied in much earlier studies, especially for essential oils and spice extractives [28]. This extensive and somewhat separate focus of attention to perfumery, combined with a rapid, seemingly disorganized development about the chemistry of food flavors has contributed to a slow evolution of a discipline-oriented identity for the field of flavors.

Although flavor substances represent an extremely wide range of chemical structures derived from almost every notable constituent of foods, the feature of "stimulating taste or aroma receptors or specialized nerves to produce an integrated psychological response known as flavor," remains as the only essential requirement for inclusion of a molecule into the flavors category of food chemistry. However, from a broader perspective, the term "flavor," has evolved to a usage that implies an overall integrated perception of all of the contributing senses (smell, taste, sight, feeling, and sound) at the time of food consumption. Thus, although the nonchemical or indirect senses (sight, sound, and feeling) often substantially influence the perception of flavors, and hence food acceptances, a discussion of these effects is beyond the scope of this chapter.

Attention in this chapter is directed toward chemicals that produce specialized odor and/or taste-related responses, but a clear distinction between the meaning of these terms and that of flavor will not always be attempted. Included will be the chemistry of important flavor systems and character-impacted compounds that have been selected to illustrate the chemistry of relevant food systems and the chemical basis for the existence of flavor compounds in foods. Where appropriate, and when information is available, structure–activity relationships for flavor compounds are noted.

Limited notice is given here to listings of profiles of flavor compounds present in various foods. Comprehensive lists of flavor compounds for foods are available elsewhere [49,84], as are tabulations of threshold concentrations for individual compounds [21]. Finally, a choice exists whether information pertaining to the flavor chemistry of major food constituents is dealt with here or in the chapters devoted to those major food constituents. It has been deemed appropriate to conduct these discussions in the major-constituent chapters. For example, many details for flavors deriving from the Maillard reaction are discussed in Chapters 3 and 14 and similarly those deriving from free radical oxidation of lipids are discussed in Chapter 4. Information on low-calorie sweeteners and on binding of flavors by macromolecules is, of necessity, partly covered here and partly covered in Chapters 3 and 5 (binding by macromolecules), and in Chapter 11 (low-calorie sweeteners).

10.1.2 Methods for Flavor Analysis (22,65)

As noted at the beginning of this chapter, flavor chemistry often has been equated with the analysis of volatile compounds using gas chromatography combined with fast-scan mass spectrometry, but this view is too restrictive as an extensive array of methods has evolved for the analysis of flavor compounds. However, only limited attention will be directed to flavor analysis here because extensive discussions can be found elsewhere [50,67,77].

Several factors make the analysis of flavors somewhat demanding, including their presence at low concentrations (ppm, 1×10^6; ppb, 1×10^9; ppt, 1×10^{12}), the complexity of mixtures (e.g., over 450 volatiles identified in coffee), the instability of some flavor compounds, and their sometimes extremely high volatility (high vapor pressures) or low volatility (low vapor pressures). Identification of flavor compounds typically requires initial isolation from the bulky constituents of foods combined with substantial concentration (e.g., distillation), but this should occur with minimal distortion of the native composition, especially when flavor quality is being studied. Adsorption of equilibrium gas-phase flavor compounds over foods onto porous polymers followed by either thermal desorption or solvent elution has provided a means to minimize destruction of sensitive compounds during isolation. However, higher-boiling compounds and some compounds present in very low concentrations may still require distillation techniques to assure adequate recovery for analysis.

Identification of flavor-active compounds and associated precursor substances is a primary goal of flavor analysis, but accurate measurement of concentrations of these compounds in foods is an equally important goal. Quantitative information is especially valuable when correlations between occurrences of flavor compounds and resulting sensory perceptions are sought. Although extensive data have been accumulated on the quantitative presence of flavor substances in headspace gases over foods and variously obtained isolates from foods, because of missing compounds or distorted quantitative values, it is often difficult to reconstruct high-quality facsimile flavors from these data.

Additionally, attempts to duplicate flavors in nutritionally modified foods (e.g., low-fat formulations) by adapting flavor compositions used in unmodified foods (e.g., full-fat formulations) also generally have met with limited successes. In response to suspicions that differing relative rates of release of individual flavor compounds from the modified food matrix (e.g., low-fat formulation) in the mouth accounted for these difficulties, considerable efforts have been devoted to the application of atmospheric pressure ionization mass spectrometry techniques for the real-time measurement of release of flavor compounds in the oral cavity. While elegant experiments have validated the time-intensity release rate concept for individual compounds from foods in the mouth, the measured differences mostly relate to flavor intensity effects, and do not seem to correlate with differences in perceived flavor qualities observed between unmodified and modified food matrix compositions. Because of these findings, attention has been shifting towards discovering flavor-modifying molecules that are present in unmodified foods (e.g., full-fat formulations) but that are excluded or omitted in modified foods (e.g., low-fat formulations).

Overall, while progress has been made in the development and application of methods that correlate objective flavor chemical data with subjective sensory information, routine assessment of flavors by purely analytical means remains limited. The development and commercialization of "electronic nose" devices [52] has been in response to the long-standing demands for rapid means to measure chemical parameters that provide reliable information about flavor intensity and quality of foods. While some successes in applications have been reported for these devices, especially in screening foods for deteriorative flavors (e.g., oxidative rancidity), they still are generally considered in early developmental stages.

10.1.3 Sensory Assessment of Flavors

Sensory assessments of flavor compounds and foods are essential for achieving objectives of flavor investigations regardless of ultimate goals. Some situations call for sensory characterization of samples by skilled individuals (experienced flavorists or researchers). However, in many instances, it is necessary to use formal sensory panels and statistical analysis to detect differences in flavors, obtain descriptive flavor information, or determine consumer flavor preferences. Excellent reviews and books are available on formal sensory analysis [1,2,62,72], and these should be consulted for detailed information on this extremely important aspect of flavor assessment.

Sensory assessments are used for documentation of qualitative flavor characteristics of flavor and aroma chemicals, and both qualitative and quantitative flavor or aroma sensations for single or

combinations of chemicals vary with the concentration. Determination of detection thresholds for flavor chemicals provides a measure of the potency of flavor provided by individual compounds, and detection threshold values are usually determined using individual representative of the general population. A range of concentrations of a selected flavor compound in a defined medium (water, milk, air, etc.) is presented to sensory panelists, and each panelist indicates whether or not the compound can be detected. The concentration range where at least half (sometimes greater) of the panelists can detect the compound is designated as the flavor threshold [21]. Compounds vary greatly in their flavor or odor potency, and thus, a minute amount of a compound having a very low threshold is perceived to have substantially greater influence on the flavor of a food than one that is quite abundant, but which possesses a high-flavor threshold.

The calculation of the odor units (OUs) involves dividing the concentration of the flavor compound by its flavor threshold (OU = concentration present/threshold concentration), and provides an estimate of the contribution by a flavor compound. More recently, aroma extract dilution analysis (AEDA) has been used extensively to identify the most potent odorants in foods [27], and this involves sensory detection of individual compounds (flavor dilution factor) in gas chromatographic effluents resulting from serial dilutions of aroma extracts from foods. Such methods provide quantitative information about the relative intensity or potency of flavor compounds present in foods and beverages. However, such determinations often exclude or grossly underestimate the qualitative flavor features of compounds. This is especially the case where individual compounds contribute the recognition, "characterizing," or "character-impact" feature to a particular flavor. These most potent aroma compound methods are criticized also because they provide flavor potency data that are determined in the absence of influences of food matrices and interactive psychophysical effects of the perception of mixtures of flavor compounds. Therefore, extrapolation of such data to actual food systems is severely limited.

10.1.4 Molecular Mechanisms of Flavor Perception

Even though understanding the molecular basis of flavor perception has long been pursued as an important goal with many practical applications, it has only been in very recent times that research discoveries in this field have begun to replace theories with established facts and principles. Much of the recent progress in understanding flavor perception has been made with the use of techniques common to contemporary molecular biology investigations.

A strong driving force for pursuing basic studies on the mechanisms of flavor perception is provided by a desire to more extensively utilize structure–activity features of molecules (e.g., a defined structural feature yields a predictable aroma or taste) to guide the development of more useful and effective flavor compounds (such as intense sweeteners; see also Chapter 11). Similarly, high demands exist for substances that mask or cancel unpleasant flavors found in some food ingredients (e.g., soy protein derivatives), and especially unwanted bitterness that is inherent to some pharmaceuticals and nutraceutical ingredients (Chapter 12).

Specialized cells of the olfactory epithelium in the nasal cavity that have the ability to detect trace amounts of volatile odorants account for the nearly unlimited variations in intensity and quality of odors and flavors. Taste buds located on the tongue and back of the oral cavity enable humans to sense sweetness, sourness, saltiness, bitterness, and umami, and these sensations contribute the taste components of food flavors. Overall, the general process of odor and taste perception at the molecular level involves three successive stages that ultimately culminate in the sensory experience of the taster. These are reception, transduction, and neural processing or coding of electrical impulse information. Detailed overviews, including schematics and descriptions, of these biological processes can be found in recent reviews [45,74].

For odorants and some of the tastants (sweet, bitter, and umami), the initial perception event involves the selective binding (believed to comply with a structural lock-and-key conceptual model) of a flavor molecule to a specific receptor protein in the membrane of an appropriate receptor cell.

When the binding of the flavor molecule to the receptor protein occurs, the chemical energy is transduced into electrical energy via one of several very specific biochemical reaction cascades.

The initial binding between a receptor protein and a flavor molecule stimulates a coupled G-protein to activate enzymic reactions yielding reaction cascade products (e.g., cyclic AMP or inositol triphosphate) that interact with and open Na^+ or Ca^{2+} ion channels in the receptor cell membrane. The resulting sudden flow of charged ions across the receptor cell membrane causes a depolarization of the cell, and produces a unique series of electrical charges (action potentials or nerve impulses) that reflect the amount of odorant stimulating the cell and provide some information about the identity of the flavor molecule. The critical processes by which electrical information is coded is largely theory at this point, but genetic and physiologic evidence supports a position that this is achieved by the production of spatial maps (differing neural firing rates and intensities) in the olfactory bulb and other brain structures.

The molecular-level events involved in the perception of sour (H^+) and salt (Na^+) ionic tastants are different from those of odorants and sweet, bitter, and umami tastants, and they are also different from each other. However, both sour and salty ionic tastants interact directly with ionic channels in taste receptor cell membranes. For sour tastants, H^+ ions bind directly to ion channels causing their closure to Na^+ flow, which then results in membrane depolarization and a neural impulse. In contrast, saltiness perception is initiated by the direct entrance of Na^+ ions from the external environment into tastant receptor cells because the ion channels are permeable to the cation of salt (Na^+). Thus, when Na^+ ions enter receptor cells and change the electrical potential across the cell membrane, the cells depolarize and generate a neural impulse in response to the presence of salt (NaCl) in the external environment.

Some flavor molecules exhibit unique sensory properties, including heat or pungency, cooling, and tingling sensations, that greatly contribute to the flavors of certain foods and beverages. Because these sensations stem from influences on certain nerve fibers and lack the involvement of specific receptor (i.e., taste or olfactory) cells in their generation, in the past they commonly have been referred to as nonspecific saporous sensations. Such sensations in oral and nasal tissues appear parallel to those detected by the cutaneous (skin) chemosensory systems (irritation, pain, heat, cold, etc.). However, to distinguish these flavor-related sensations emanating from the innervating systems of the oral and nasal cavities (i.e., trigeminal, glossopharyngeal, and vagus), recently the term, chemesthesis, has been coined to collectively refer to these saporous sensations.

Other nonspecific, chemically induced, flavor-influencing sensations (fullness, complexity, etc.) apparently are sensed by the trigeminal neural system, but the compounds causing the effects are not widely known and the mechanisms of perception are not clearly understood.

Advances in unraveling the details of the intriguing process of flavor perception are still actively emerging, and reviews on the topic can be found elsewhere [51,74].

10.2 TASTE AND OTHER SAPOROUS SUBSTANCES

Frequently, although not always, substances responsible for these aspects of flavor perception are water soluble and relatively nonvolatile. As a general rule, they are also present at higher concentrations in foods than those responsible for aromas, and they have been often treated lightly in coverages of flavors. Because of their extremely influential role in the acceptance of foods, the chemistry of substances responsible for taste sensations as well as those responsible for some of the less defined flavor sensations are examined here.

10.2.1 Sweet Taste Substances

Sweet substances have been the focus of much attention because of interest in sugar alternatives and the desire to find suitable replacements for certain low-calorie sweeteners, including saccharin and

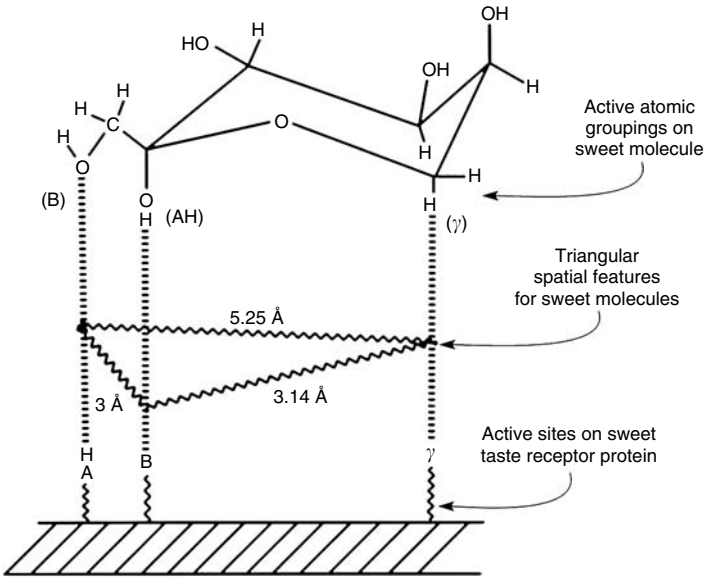
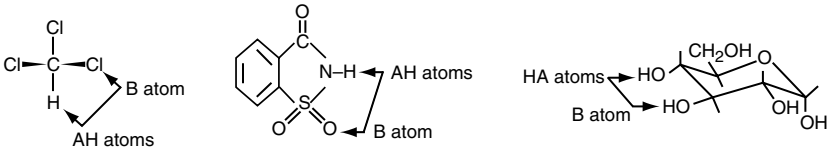


FIGURE 10.1 Schematic showing the relationship between AH/B and γ sites in the saporous sweet unit for β-D-fructopyranose.

cyclamate (see Chapter 11). Before modern sweetness theories were advanced, it was popular to deduce that sweetness was associated with hydroxyl (—OH) groups because sugars molecules are dominated by this feature. However, this view was soon subject to criticism because polyhydroxy compounds vary greatly in sweetness, and many amino acids, some metallic salts, and unrelated compounds, such as chloroform ($CHCl_3$) and saccharin (Chapter 11), are also sweet. Still, it was apparent that some common characteristics existed among sweet substances, and over the past 75 years a theory-relating molecular structure and sweet taste has evolved that satisfactorily explains why certain compounds exhibit sweetness.

Shallenberger and Acree [69] first proposed the AH/B theory for the saporous (taste eliciting) unit common to all compounds that cause a sweet sensation (Figure 10.1). The saporous unit was initially viewed as a combination of a covalently bound H-bonding proton and an electronegative orbital positioned at a distance of about 3 Å from the proton. Thus, vicinal electronegative atoms on a molecule are essential for sweetness. Further, one of the atoms must possess a H-bonding proton. Oxygen, nitrogen, and chlorine atoms frequently fulfill these roles in sweet molecules, and hydroxyl group oxygen atoms can serve either the AH or B function in a molecule. Simple AH/B relationships are shown for chloroform (I), saccharin (II), and glucose (III).



(I) Chloroform (II) Saccharin (III) Glucose

As indicated in Figure 10.1, however, stereochemical requirements are also imposed on the AH/B components of the saporous unit so that they will align suitably with the receptor site. The interaction between the active groups of the sweet molecule and the taste receptor is currently envisioned to occur through H-bonding of the AH/B components to similar structures in the taste receptor. A third feature

also has been added to the theory to extend its validity to intensely sweet substances. This addition incorporates appropriate stereochemically arranged lipophilic regions of sweet molecules, usually designated as γ that are attracted to similar lipophilic regions of the taste receptor. Lipophilic portions of sweet molecules are frequently methylene ($-CH_2-$), methyl ($-CH_3$), or phenyl ($-C_6H_5$) groups. The complete sweet saporous structure is geometrically situated so that triangular contact of all active units (AH, B, and γ) with the receptor molecule occurs for intensely sweet substances, and this arrangement forms the rationale for the tripartite structure theory of sweetness.

The γ-site is an extremely important feature of intensely sweet substances, but plays a lesser role in sugar sweetness [8]. It appears to function through facilitating the accession of certain molecules to the taste receptor site, and as such affects the perceived intensity of sweetness. Since sugars are largely hydrophilic, this feature comes into play in a limited sense only for some sugars, such as fructose. This component of the saporous sweet unit probably accounts for a substantial portion of the variation in sweetness quality that is observed between different sweet substances. Not only it is important in the time-intensity or temporal aspects of sweetness perception, but it also appears to relate to some of the interactions between sweet and bitter tastes observed for some compounds.

Sweet–bitter sugar structures possess features that apparently allow them to interact with either or both types of receptors, thus producing the combined taste sensation. Bitterness properties in structures depress sweetness even if the concentration in a test solution is below that for the bitter sensation. Bitterness in sugars appears to be imparted by a combination of effects involving the configuration of the anomeric center, the ring oxygen, the primary alcohol group of hexoses, and the nature of any substituents. Often, changes in the structure and stereochemistry of a sweet molecule lead to the loss or suppression of sweetness or the induction of bitterness.

10.2.2 Bitter Taste Substances (59,60)

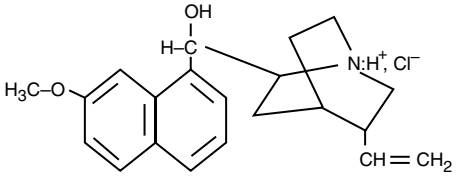
The bitterness sensation appears to be closely related to sweetness from a molecular structure–receptor relationship. Bitterness resembles sweetness because of its dependence on the stereochemistry of stimulus molecules, and the two sensations are triggered by similar features in molecules, causing some molecules to yield both bitter and sweet sensations. Although sweet molecules must contain two polar groups that may be supplemented with a nonpolar group, bitter molecules appear to have a requirement for only one polar group and a hydrophobic group [9].

However, some [5,7,14] believe that most bitter substances possess an AH/B entity identical to that found in sweet molecules as well as the hydrophobic group. In this concept, the orientation of AH/B units within specific receptor sites, which are located on the flat bottom of receptor cavities, provides the discrimination between sweetness and bitterness for molecules possessing the required molecular features. Molecules that fit into sites that were oriented for bitter compounds give a bitter response; those fitting the orientation for sweetness elicit a sweet response. If the geometry of a molecule were such that it could orient in either direction, it would give bitter–sweet responses.

Such a model appears especially attractive for amino acids where D-isomers are sweet and L-isomers are bitter [43]. Since the hydrophobic or γ-site of the sweet receptor is nondirectional lipophilicity, it could participate in either sweet or bitter responses. Molecular bulkiness factors serve to provide stereochemical selectivity to the receptor sites located in each receptor cavity. It can be concluded that there is a very broad structural basis for the bitter taste modality, but most empirical observations about bitterness and molecular structure can be explained by current theories.

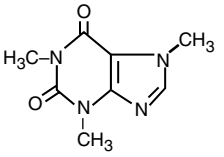
Quinine is an alkaloid that is generally accepted as the standard for the bitter taste sensation. The detection threshold for quinine hydrochloride (IV) is about 10 ppm. In general, bitter substances have lower taste thresholds than other taste substances, and they also tend to be less soluble in water than other taste-active materials. Quinine is permitted as an additive in beverages, such as soft drinks that also have tart-sweet attributes. The bitterness blends well with the other tastes, and provides

a refreshing gustatory stimulation in these beverages. The practice of mixing quinine into soft drink beverages apparently stems from efforts to suppress or mask the bitterness of quinine when it was prescribed as a drug for malaria.

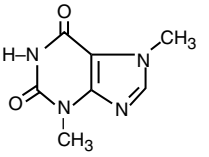


(IV) Quinine hydrochloride

In addition to soft drinks, bitterness is an important flavor attribute of several other beverages consumed in large quantities, including coffee, cocoa, and tea. Caffeine (V) is moderately bitter at 150–200 ppm in water, and occurs in coffee, tea, and cola nuts. Theobromine (VI) is very similar to caffeine, and is present most notably in cocoa, where it contributes to bitterness. Caffeine is added in concentrations up to 200 ppm to soft cola beverages, and much of the caffeine employed for this purpose is obtained from extractions of green coffee beans that are carried out in the preparation of decaffeinated coffee.



(V) Caffeine



(VI) Theobromine

Large amounts of hops are employed in the brewing industry to provide unique flavors to beer. Bitterness, contributed by some unusual isoprenoid-derived compounds, is a very important aspect of hop flavor. These nonvolatile, bitter substances can generally be categorized as derivatives of humulone or lupulone, that is, α- or β-acids, respectively, as they are known in the brewing industry. Humulone is the most abundant substance, and it is converted during wort boiling to isohumulone by an isomerization reaction (Figure 10.2) [16].

Isohumulone is the precursor for the compound that causes the sunstruck or skunky flavor in beer exposed to light. In the presence of hydrogen sulfide from yeast fermentation, a photocatalyzed reaction occurs at the carbon adjacent to the keto group in the iso-hexenyl chain. This gives rise to 3-methyl-2-butene-l-thiol (prenylmercaptan) that has a skunky aroma. Selective reduction of the ketone

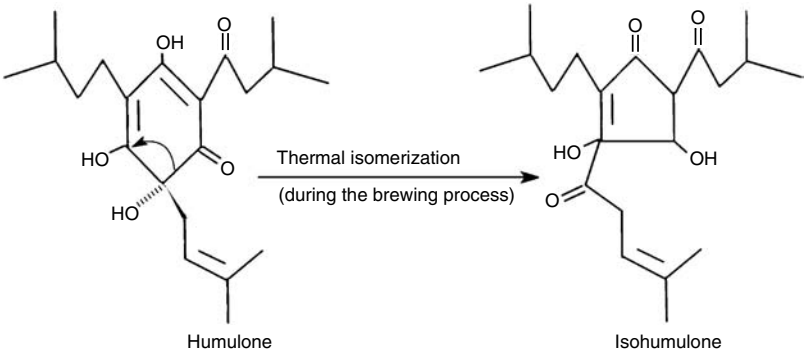
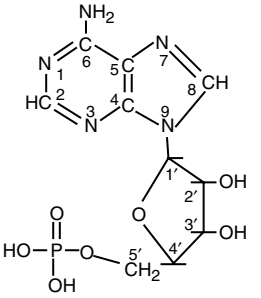


FIGURE 10.2 Thermal isomerization of humulone to isohumulone occurring during wort boiling in the traditional brewing process.

in preisomerized hop extracts prevents this reaction, and permits packaging of beer in clear glass without the development of the skunky or sunstruck flavor. Whether volatile hop aroma compounds survive the wort-boiling process was a controversial topic for a number of years. However, it is now well documented that influential compounds do survive the wort-boiling process and others are formed from hop bitter substances; together they contribute to the kettle-hop aroma of beer.

Although bitterness is desirable in many foods and beverages, unwanted bitterness in some foods and beverages, including novel nutraceutical ingredients and pharmaceutical preparations, is frequently a problem. Extensive efforts have been expended to identify substances that mask bitter flavors, but to date these have met with limited successes. While certain gums or viscosity-enhancing polymers suppress bitter flavors, generally their use only partly alleviates the problem. However, recently it has been discovered that adenosine-5′-monophosphate (AMP) (VII) that is associated with intermediary energy metabolism possesses potent bitter-blocking properties, and its application in bitter flavor suppressions appears promising.



(VII) Adenosine-5′-monophosphate
(AMP; a potent bitter blocker)

The development of excessive bitterness is a major problem of the citrus industry, especially in processed products. In the case of grapefruit, some bitterness is desirable and expected, but frequently the intensity of bitterness in both fresh and processed fruits exceeds that preferred by many consumers. Citrus fruits contain several of flavonone glycosides, and naringen is the predominant flavonone found in grapefruit and bitter orange (*Citrus auranticum*). Juices that contain high levels of naringen are extremely bitter, and are of little economic value except in instances where they can be extensively diluted with juices containing low bitterness levels. The bitterness of naringen is associated with the configuration of the molecule that develops from the 1 to 2-linkage between rhamnose and glucose. Naringenase is an enzyme that has been isolated from commercial citrus pectin preparations and from *Aspergillus* sp., and this enzyme hydrolyzes the 1 to 2-linkage (Figure 10.3) to yield nonbitter products. Immobilized enzyme systems have also been developed to debitter grapefruit juices containing excessive levels of naringen. Naringen has also been commercially recovered from grapefruit peels and is used instead of caffeine for bitterness in some food applications.

The principal bitter component in navel and Valencia oranges is a triterpenoid dilactone (A- and D-rings) called limonin, and it is also found as a bittering agent in grapefruit. Limonin is not present to any extent in intact fruits, but rather a flavorless limonin derivative produced by enzymic hydrolysis of limonin's D-ring lactone is the predominant form (Figure 10.4). After juice extraction, acidic conditions favor the closing of the D-ring to form limonin, and the phenomenon of delayed bitterness occurs, yielding serious economic consequences.

Methods for debittering orange juice have been developed using immobilized enzymes from *Arthrobacter* sp. and *Acinetobacter* sp. [34]. Enzymes that simply open the D-ring lactone provide only temporary solutions to the problem because the ring closes again under acidic conditions. However, the use of limonoate dehydrogenase to convert the open D-ring compound to nonbitter 17-dehydrolimonoate A-ring lactone (Figure 10.4) provides an irreversible means to debitter orange

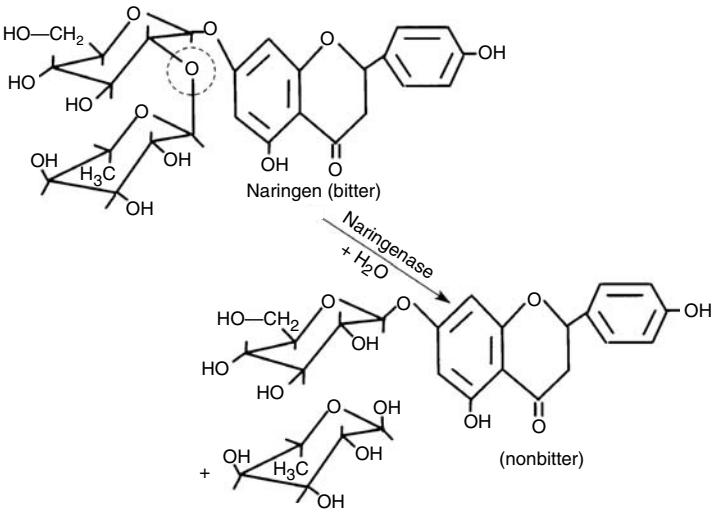


FIGURE 10.3 Reaction showing the enzymatic hydrolysis of naringen by naringenase that is used in the debittering of citrus products.

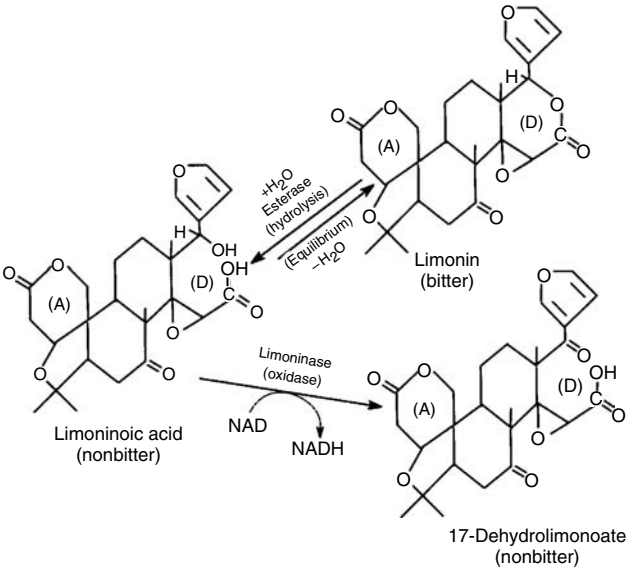


FIGURE 10.4 Equilibrium reaction leading to the formation limonin, and enzymic debittering reactions that reverse bitterness development in citrus juices.

juice. Methods to debitter citrus juices also include the use of polymeric adsorbants that currently are the preferred methods for commercial processors [40].

Pronounced, undesirable bitterness is frequently encountered in protein hydrolysates and aged cheeses, and this effect is caused by the overall hydrophobicity of amino acid side chains in peptides. All peptides contain suitable numbers of AH-type polar groups that can fit the polar receptor site, but individual peptides vary greatly in the size and nature of their hydrophobic groupings and thus in the ability of these hydrophobic groups to interact with the essential hydrophobic sites of bitterness receptors. Ney [59] has shown that the bitter taste of peptides can be predicted by a calculation of a

TABLE 10.1
Calculated ΔG Values for Individual Amino Acids

Amino Acids	ΔG Value[a] (kJ mol^{-1})
Glycine	0 (0)
Serine	167.3 (40)
Threonine	1839.9 (440)
Histidine	2090.8 (500)
Aspartic acid	2258.1 (540)
Glutamic acid	2299.9 (550)
Arginine	3052.6 (730)
Alanine	3052.6 (730)
Methionine	5436.1 (1300)
Lysine	6272.4 (1500)
Valine	7066.9 (1690)
Leucine	10119.5 (2420)
Proline	10955.8 (2620)
Phenylalanine	11081.2 (2650)
Tyrosine	12001.2 (2870)
Isoleucine	12419.4 (2970)
Tryptophan	12544.8 (3000)

[a] ΔG values in calories mol^{-1} are shown in parentheses; 1 calories = 4.1816 kJ. ΔG values represent free energy change for the transfer of amino acid side chains from ethanol to water. These values are slightly different from those obtained from transfer of amino acid side chains from octanol to water (see Table 5.3).

Source: From Ney, K. H. (1979). In *Food Taste Chemistry* (J. C. Boudreau, ed.), American Chemical Society, Washington, D.C., pp. 149–173.

mean hydrophobicity value, termed Q. The ability of a protein to engage in hydrophobic associations is related to the sum of the individual hydrophobic contributions of the nonpolar, amino acid side chains, and these interactions contribute mainly to the free energy (ΔG) associated with protein unfolding. Thus, by summation of ΔG values for the individual amino acid side chains in a peptide, it is possible to calculate the mean hydrophobicity Q using Equation 10.1:

$$Q = \frac{\sum \Delta G}{n}, \tag{10.1}$$

where n = number of amino acid residues. Individual ΔG values for amino acids have been determined from solubility data [76], and these are summarized in Table 10.1. Q-values above 5855 based on joules mol^{-1} (1400 based on calories mol^{-1}) indicate that the peptide will be bitter; values below 5436 based on joules mol^{-1} (1300 based on calories mol^{-1}) assure that the peptide will not be bitter. The molecular weight of a peptide also influences its ability to produce bitterness, and only those with molecular weights below 6000 have a potential for bitterness. Peptides larger than this apparently are denied access to receptor sites because of their bulkiness (see Chapter 5).

The peptide shown in Figure 10.5 is derived from the cleavage of α_{s1}-casein between residue 144–145 and residue 150–151 [59], and has a calculated Q-value of 9576 based on joules mol^{-1} (2290

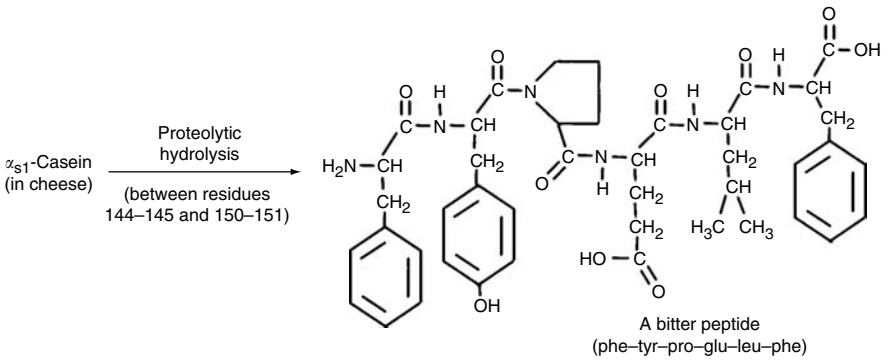
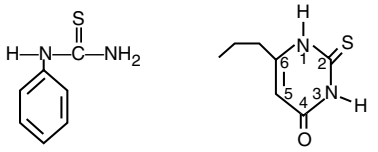


FIGURE 10.5 Reaction depicting the hydrolysis of α_{s1}-casein to form a bitter peptide (phe–tyr–pro–glu–leu–phe) that exhibits strong overall nonpolar features.

based on calories mol^{-1}). This peptide is very bitter, and is illustrative of the strongly hydrophobic peptides that can be derived readily from α_{s1}-casein. Such peptides are responsible for bitterness that develops in aged cheeses.

Because of genetic differences in humans, individuals vary in their ability to perceive bitter substances. At a defined concentration, certain substances may be bitter, bitter–sweet or tasteless depending on the individual. Saccharin is perceived as purely sweet by some individuals, but others find it to range from only slightly bitter and sweet to quite bitter and sweet. Many other compounds also show marked variations in the manner in which individuals perceive them, and frequently either taste bitter or are not perceived at all.

Phenylthiocarbamide or PTC (VIII) is one of the most notable compounds in this category [1], and it was discovered in the early 1930s [4] that for this compound about 40% of the Caucasian American population taste is blind to the bitterness taste attribute that is perceived by the other 60% of the Caucasian American population. Because the ability to perceive bitterness of PTC was so clearly genetically controlled, its use as a marker for exploring behaviorial and metabolic differences between bitter tasters and nontasters soon followed. Because PTC also exhibits a sulfurous odor, more recently researchers have largely adopted 6-*n*-propyl-2-thio-uracil (PROP) (IX) that does not have an inherent odor for continued studies [63]. For these two molecules, the N—C═S grouping that is believed to be responsible for their bitter tastes.



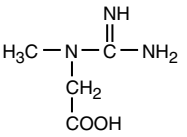
(VIII) Phenylthiocarbamide (PTC)

(IX) 6-*n*-Propyl-2-thiouracil (PROP or PRU)

Recent studies have revealed that individuals who find PROP to be intensely bitter are genetically endowed with the ability to perceive many flavors in an intensified fashion, and these individuals have become known as "supertasters." Currently, researchers are investigating many physiological and psychological aspects of both PROP-insensitive and sensitive individuals in hopes that underlying factors governing food intake and preference as well as certain pathological conditions and health risks in the general population can be discovered.

Although PTC and PROP are novel compounds that do not occur in foods, creatine (X) is a constituent of muscle foods that exhibits similar properties of varied bitter taste sensitivity in the

population. Creatine may occur at levels up to about mg/g in lean meats [1], and this is adequate to make some soups taste bitter to sensitive individuals.



(X) Creatine

Bitterness occurs in salts, and this sensory property greatly hampers the substitution of alternate cations for sodium in foods compositionally modified to permit restricted sodium intake by consumers. The atomic (molecular) features causing bitterness in salts apparently are quite different from those that cause bitterness in organic compounds. Bitterness in salts seems to be related to the sum of the ionic diameters of the anion and cation components comprising the salt in question [5,6]. Salts with ionic diameters below 6.5 Å are purely salty in taste (LiCl = 4.98 Å; NaCl = 5.56 Å; KCl = 6.28 Å) although some individuals find KCl to be somewhat bitter. As the ionic diameter increases (CsCl = 6.96 Å; CsI = 7.74 Å) salts become increasingly bitter. Magnesium chloride (8.50 Å) is therefore quite bitter.

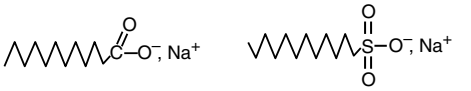
10.2.3 Salty Taste Substances

Classic, clean salty taste is provided by sodium chloride (NaCl), but it is also given by lithium chloride (LiCl) which cannot be used in foods because of its toxic properties. In general, salts exhibit complex tastes usually described as consisting of psychological mixtures of classic sweet, bitter, sour, and salty perceptual components. However, the tastes of salts often fall outside the traditional taste sensations [66] and are difficult to describe in the classic flavor terms. In some instances other vague terms, such as chemical or soapy, often seem to more accurately describe the sensations produced by salts than do the classic terms.

The flavor effects of NaCl appear to extend well beyond those of the classic tastes, and in food applications NaCl clearly provides special flavor-enhancing properties. These effects can be easily demonstrated through the substantial reduction or omission of NaCl from standard food formulations (e.g., bread and bakery items).

It has been established that cations cause the basic salty taste, and that anions modify the basic salt taste [3]. Sodium and lithium cations produce only salty taste while potassium and other alkaline earth cations produce a combination of both salty and bitter tastes. Anions modify salt tastes by inhibiting the tastes of cations, and they also frequently contribute tastes of their own. Among the anions commonly found in foods, the chloride anion is least inhibitory to the salty taste, and the citrate anion is more inhibitory than orthophosphate anions. Furthermore, the chloride anion does not contribute a taste, and the citrate anion contributes less taste than the orthophosphate anion.

Anion taste effects impact the flavor of many foods, such as processed cheese, where citrate and phosphate anions contained in emulsifying salts (Chapter 11) suppress perceived saltiness contributed by sodium ions and also add anion tastes. Similarly, soapy tastes caused by sodium salts of long-chain fatty acids (XI) and detergents or long-chain sulfates (XII) result from specific tastes elicited by the anions, and these tastes can completely mask the taste of the cation.



(XI) Sodium laurate (XII) Sodium lauryl sulfate

National policies encouraging reduction in sodium consumption have stimulated interest in foods in which sodium salts have been replaced by alternative substances, particularly those containing

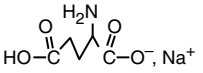
potassium and ammonium ions. Since foods flavored with these substitutes have different, usually less desirable, tastes than those flavored with NaCl, renewed efforts are being expended to better understand the basic mechanisms of the salty taste and in the development of salt substitutes in the hope that low-sodium products with near normal salty taste can be devised.

10.2.4 Sour Taste Substances

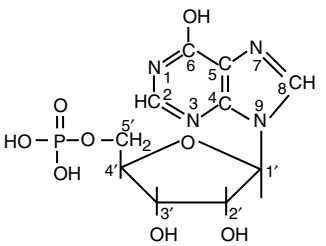
Sour taste substances are acidic in nature, and thus contain at least one proton that is dissociable in aqueous systems (Chapter 10.11). Although the initial molecular level event in the perception of acidic, sour, or tart flavors involves the binding of protons (H^+) to receptor cell membrane ion channels that results in closure to Na^+ flow and depolarization, the qualitative aspects of the sour taste response are poorly understood. Contrary to popular belief, the acid strength in a solution does not appear to be the major determinant of the sour sensation; rather, other poorly understood molecular features appear to be of primary importance (e.g., molecular weight, size, and overall polarity), and prior empirical experience often determines the selection of acids for applications in foods.

10.2.5 Umami Taste Substances (39)

Compounds eliciting this taste sensation have been utilized by humans to improve flavors since the inception of food cooking and preparation. For many years, umami tastants, notably monosodium L-glutamate (MSG; XIII) and the 5′-ribonucleotides (inosine-5′-monophosphate; 5′-IMP; XIV; and guanosine-5′-monophosphate), were scientifically relegated to the category of nonspecific taste responses because specific taste receptors for these substances had not yet been detected. However, since the discovery of taste receptors for these compounds, umami is now widely accepted as a basic taste response [43].



(XIII) Monosodium L-glutamate (MSG; umami)



(XIV) Inosine-5′-monophosphate (IMP; umami)

Umami substances contribute a delicious, mouthwatering taste to foods when used at levels in excess of their independent detection threshold, and they modify and enhance flavors at levels below their independent detection thresholds. Their effects are prominent and desirable in the flavors of vegetables, meats, poultry, fish, shellfish, and aged cheeses.

D-Glutamate and the 2′- or the 3′-ribonucleotides do not exhibit flavor-enhancing activity. Several synthetic derivatives of the 5′-ribonucleotides have strong flavor-enhancing properties [44]. Generally, these derivatives have substitutions on the purine moiety in the 2-position. A synergistic interaction occurs between MSG and the 5′-ribonucleotides in providing both the umami taste and in enhancing flavors, and mixtures of these substances are widely used commercially. There is some evidence to indicate that some of the flavor-enhancing properties of umami compounds result from their joint occupancy of receptor sites involved in perception of sweet, sour, salty, and bitter sensations.

Although MSG and 5′-IMP and 5′-guanosine monophosphate are the only flavor enhancers used commercially, 5′-xanthine monophosphate and a few natural amino acids, including L-ibotenic

acid and L-tricholomic acid, are potential candidates for commercial use [89]. Much of the flavor contributed to foods by yeast hydrolysates results from the 5′-ribonucleotides present. Large amounts of purified flavor enhancers employed in the food industry are derived from microbial sources, including phosphorylated (*in vitro*) nucleosides derived from RNA [44]. Discussions on general flavor enhancement can be found in several reviews [44,89].

10.2.6 Kokumi Taste Substances and Other Flavor Modifiers

As mentioned in Section 10.2.3, common salt (NaCl) provides profound flavor enhancing and modifying effects to many food flavors. Although salt is specifically sensed by specialized taste cells (Section 10.1.2), many believe that it also may provide flavor enhancing by modifying the functions of other basic taste receptor cells or through other sensations emanating from other neural systems (e.g., trigeminal, etc.) in the oral cavity. Thus, salt likely possesses some properties similar to other flavor-enhancing substances whose overall taste-modifying functions remain poorly understood.

The Japanese have introduced a separate term, kokumi, to refer to at least part of these chemicals which do not elicit responses for the original four basic tastes or the umami response, but which enhance food palatability by providing what is best described as fullness, complexity, continuity, thickness, and body to food flavors [81]. For example, the principal precursors for the characterizing volatile aroma compounds in garlic and onions are *S*-substituted cysteine sulfoxide amino acids (Figure 10.6), and both of these compounds are readily water soluble and they exhibit strong kokumi properties that distinctly influence palatability [81,82]. Thus, although flavors of foods containing garlic (e.g., pasta sauces, sautéed meats, etc.) may not exhibit readily distinguishable volatile garlic flavor notes, their flavors are perceived as extremely complex, full, and palatable because of the presence of *S*-(2-propenyl)-L-cysteine sulfoxide.

Although readily water-soluble substances reported to provide kokumi flavors are not yet numerous, another cysteine-containing peptide, glutathione (Figure 10.6), also is kokumi active [83]. In a potentially related response, succinic acid (and its soluble salts; Figure 10.6) exhibits a distinct broth-like flavor characteristic in addition to a sour taste. Although the flavor of succinic acid has not

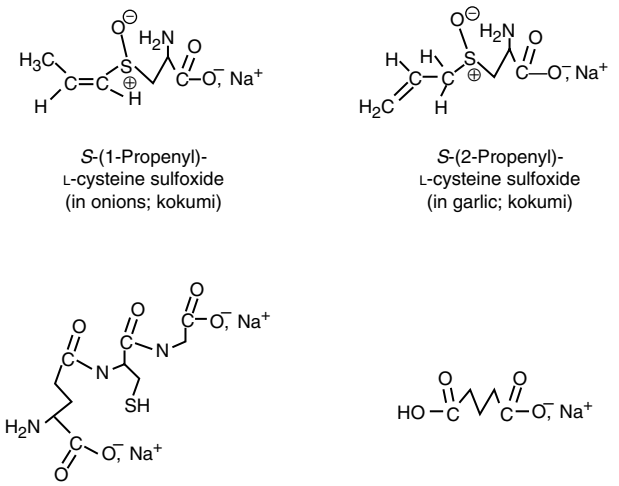


FIGURE 10.6 Structures of some readily water-soluble, flavor-modifying compounds.

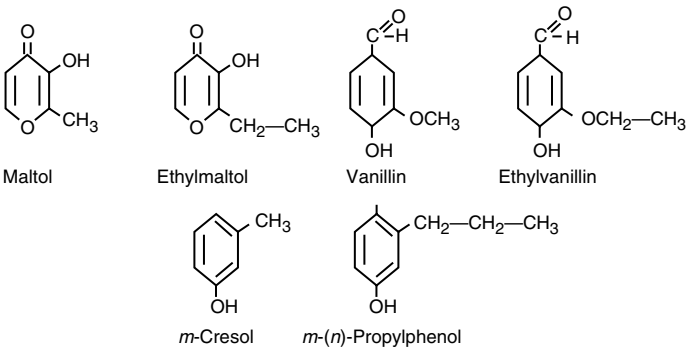


FIGURE 10.7 Structures of some flavor-modifying compounds with limited water solubility.

been classified kokumi as yet, this substance is used commercially to provide a brothy complexity to savory flavors, especially in meat-type sauces.

A number of other descriptive terms are used to describe a variety of flavor modifications that appear to be kokumi-related influences, and these include velvety, richness, creaminess, and juiciness. A number of both natural and synthetic substances (Figure 10.7) possess the ability to provide such modifications to flavors, and some structural similarities occur among some of these substances. Of these, vanillin-type flavors comprise one of the most popular flavors worldwide, and the aromas of vanillin and ethylvanillin are perceived as extremely desirable by most. However, in addition to aroma contributions, vanillin-related substances also provide flavor-modifying effects that result in enhanced smoothness, richness, and creaminess flavor sensations, particularly in sweet, fat-containing foods such as ice cream.

Similarly, maltol and ethylmaltol (Figure 10.7) also have been used widely as commercial flavor enhancers for sweet goods and fruit-containing products. Although both of these substances possess pleasant, burnt caramel aromas at high concentrations, they often are marketed for the smooth, velvety sensation they impart to sweet goods and fruit juices and products at relatively low levels (ca 50 ppm) at which concentrations the caramelic notes are not distinguishable. Ethylmaltol is more effective as a sweetness enhancer than maltol, but maltol still generally lowers the detection threshold concentration for sucrose by a factor of 2.

Recently, some alkylphenols that occur naturally in the milk and meat of ruminants been found to contribute mouthcoating, richness enhancing, and juiciness sensations at very low concentrations (ng/g). *m*-Alkylsubstitution on the aromatic ring provides the most influential flavor-modifying effects among the members of this group, and *m*-cresol and *m*-(*n*)-propylphenol (Figure 10.7) are the most important in bovine-derived products and ingredients [32].

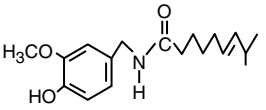
10.2.7 Pungent Substances

Pungency is a chemesthetic property exhibited by a number of compounds found in spices and vegetables that cause characteristic hot, sharp, and stinging sensations [15]. Some pungent principles, such as those found in chili peppers, black pepper, and ginger, are not volatile and unless made airborne via aerosol droplets, exert their effects principally on oral tissues. Other spices and vegetables contain pungent principles that are somewhat volatile, and produce both pungency in the oral and nasal cavities as well as characteristic aromas. These include mustards, horseradish, vegetable radishes, watercress, onions, and the aromatic spice, clove, which contains eugenol as the active component.

All of these pungent spices and vegetables are used in foods to provide characteristic flavors or to generally enhance palatability. Usage at low concentrations in processed foods frequently provides a liveliness to flavors through subtle contributions that fill out the perceived flavors. Only the three

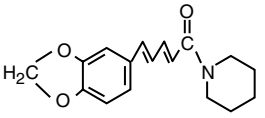
major pungent spices, chili peppers, ginger, and pepper, are discussed in this section of the chapter, but others are mentioned later in discussions about plant-derived flavor systems (i.e., isothiocyanates, thiopropanal-*S*-oxide, and eugenol). Comprehensive reviews on pungent compounds are also available (cf. [24]).

Chili peppers (*Capsicum* sp.) contain a group of substances known as capsaicinoids, which are vanillylamides of monocarboxylic acids with varying chain length (C_8–C_{11}) and unsaturation. Capsaicin (XV) is representative of these pungent principles. Several capsaicinoids containing saturated straight-chain acid components are synthesized as substitutes for natural chili extractives or oleoresins. The total capsaicinoid content of world *Capsicum* sp. vary widely [24]; for example, red pepper contains 0.06%, cayenne red pepper, 0.2%, Sannam (India), 0.3%, Uganda (Africa), 0.85%. Sweet paprika contains a very low concentration of pungent compounds and is used mainly for its coloring effects and subtle flavors in part derived from carotenoid oxidation. Chili peppers also contain a number of volatile aroma compounds that become part of the overall flavor of foods seasoned with them.



(XV) Capsaicin

Black and white pepper are made from the berries of *Piper nigrum*, and differ only in that black pepper prepared from immature, green berries and white pepper is made from more mature berries usually harvested at the time they are changing from green to yellow in color, but before they become red. The principal pungent compound in pepper is piperine (XVI), an amide. The *trans*-geometry of the alkyl unsaturation is necessary for strong pungency, and loss of pungency during exposure to light and storage is attributed mainly to isomerizations to *cis* forms of these double bonds [24]. Pepper also contains volatile compounds, including L-formylpiperdine and piperonal (heliotropin), which contribute to flavors of foods seasoned with pepper spice or oleoresins. Piperine is also synthesized for use in flavoring foods.



(XVI) Piperine

Ginger is a spice derived from the rhizome of a tuberous perennial, *Zingiber officinale* Roscoe, and it possesses pungent principles as well as some volatile aroma constituents. The pungency of fresh ginger is caused by a group of phenylalkyl ketones called gingerols, and [6]-gingerol (Figure 10.8)

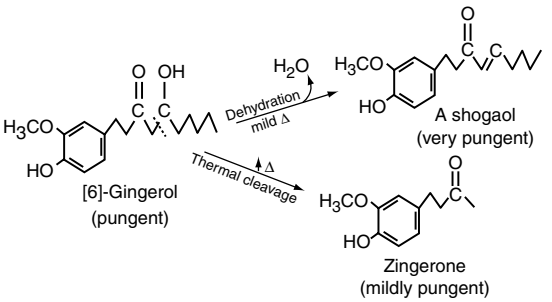
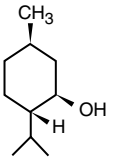


FIGURE 10.8 Reactions altering gingerol that affect the relative pungency of ginger.

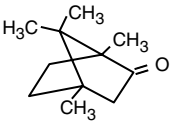
is most active of these compounds. Gingerols vary in chain-length (C_5–C_9) external to the hydroxyl-substituted C atom. During drying and storage gingerols tend to dehydrate to form an external double bond that is in conjugation with the keto group. The reaction results in a group of compounds known as shogaols that are even more potent pungent compounds than the gingerols. Exposure of [6]-gingerol to substantially elevated temperatures can lead to cleavage of the alkyl chain external to the keto group, yielding a methyl ketone, zingerone that exhibits only mild pungency.

10.2.8 Cooling Substances (85)

Cooling is another chemesthetic sensation that occurs when certain chemicals contact the nasal or oral tissues and stimulate the nonspecific neural systems (e.g., trigeminal, etc.). These effects when caused by naturally occurring substances are most commonly associated with mint-like flavors, including peppermint, spearmint, and wintergreen. Several compounds cause the sensation, but (−)-menthol (XVII), in the natural form (L-isomer), is most commonly used in flavors. A number of synthetic cooling compounds have been discovered, and both natural and synthetic compounds often also produce an accompanying camphoraceous aroma. Camphor (XVIII) is cited often as the model for camphoraceous group of compounds because it produces a distinctive odor in addition to a cooling sensation.



(XVII) (—)-Menthol



(XVIII) D-Camphor

The cooling effect produced by the mint-related compounds is mechanistically different from the slight cooling sensation produced when polyol sweeteners (Chapters 3 and 11), such as xylitol, are tasted as crystalline materials. In the latter case, it is generally believed that an endothermic dissolution of the materials gives rise to the effect.

10.2.9 Astringent Substances

Astringency is a feeling-related phenomenon perceived as a dryness in the mouth along with a coarse puckering of the oral tissue [46]. Astringency usually results from the association of tannins or polyphenols (Chapter 9) with proteins in the saliva to form precipitates or aggregates. Additionally, sparingly soluble proteins, such as those found in certain dry milk powders, can also combine with proteins and mucopolysaccharides of saliva and cause astringency. Astringency is often confused with bitterness because many individuals do not clearly understand its nature, and many polyphenols or tannins cause both astringent and bitter sensations, which is the case for red wines [1].

The more astringent tannins are often condensed tannins resulting from oxidative reactions, and these molecules offer broad cross-sectional areas (Figure 10.9) suitable for hydrophobic associations with proteins. Tannins contain many phenolic groups that can convert to quinoid structures, and these in turn can cross-link chemically with proteins [58]. Such cross-links have been suggested as a possible contributor to astringency activity.

Astringency may be a desirable flavor property, such as in tea. However, the practice of adding milk or cream to tea suppresses astringency through binding of polyphenols with milk proteins. Red wine is a good example of a beverage that exhibits both astringency and bitterness caused by polyphenols or tannins. However, too much astringency is considered sensorily undesirable in wines, and means are often taken to reduce polyphenol tannins that are related to the anthocyanin pigments.

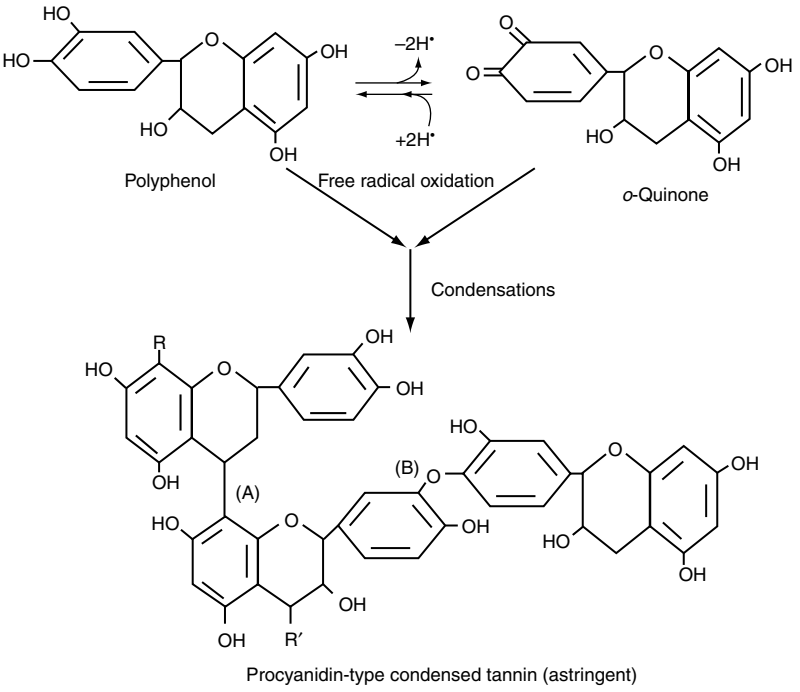


FIGURE 10.9 Model reaction illustrating the formation of a procyanidin-type tannin with large planar hydrophobic areas capable of associating with proteins to cause astringency.

Astringency derived from polyphenols in unripe bananas can also lead to an undesirable taste in products to which such bananas have been added [23].

10.3 VEGETABLE, FRUIT, AND SPICE FLAVORS (11,64)

Categorization of vegetable and fruit flavors in a reasonably small number of distinctive groups is not easy since logical groupings are not necessarily available for vegetables and fruits. For example, some information on plant-derived flavors was presented in the section on pungency, and some are covered in the section dealing with the development of "reaction" flavors. Emphasis in this section is on the biogenesis and development of flavors in important vegetables and fruits. For information on other fruit and vegetable flavors, the reader is directed to the general references (cf. [49]).

10.3.1 Sulfur-Containing Volatiles in *Allium* sp.

Plants in the genus *Allium* are characterized by strong, penetrating aromas, and important members are onions, garlic, leek, chives, and shallots. These plants lack the strong characterizing aroma unless the tissue is damaged and enzymes are decompartmentalized so that the flavor precursors can be converted to odorous volatiles. In the case of onions (*A. cepa* L.), the precursor of the sulfur compounds that are responsible for the volatile flavor and aroma of this vegetable is *S*-(1-propenyl)-L-cysteine sulfoxide [70,86], and this substance also possesses kokumi flavor properties (Section 10.2.6). This substituted cysteine sulfoxide precursor is also found in leek.

Rapid hydrolysis of *S*-(1-propenyl)-L-cysteine sulfoxide by allinase in onions yields an unstable sulfenic acid intermediate along with ammonia and pyruvate (Figure 10.10). The sulfenic acid undergoes further rearrangements to yield the lachrymator, thiopropanal-*S*-oxide that is also associated with the overall aroma of fresh onions. The pyruvic acid produced by the enzymatic conversion of

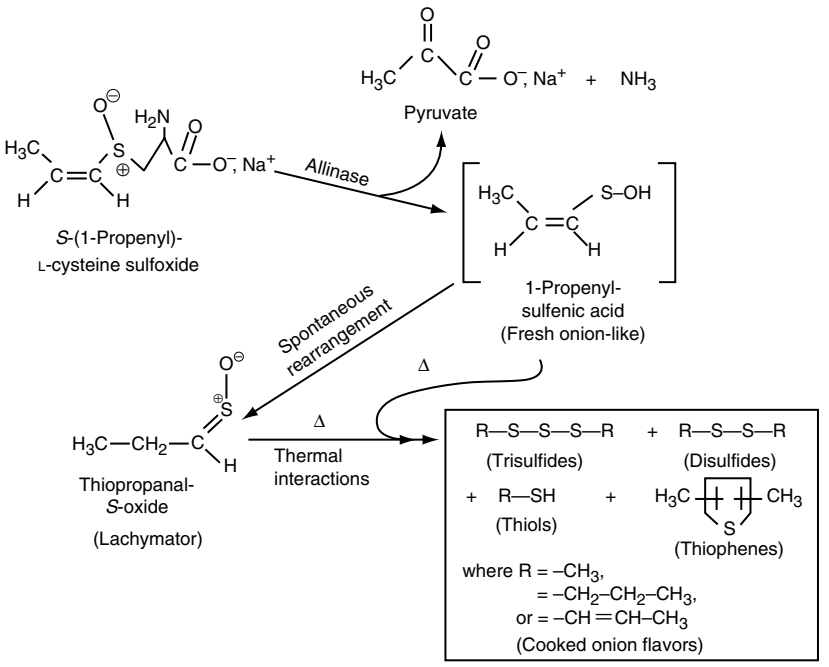


FIGURE 10.10 Reactions involved in the formation of onion flavors.

the precursor compound is a stable product of the reaction, and serves as a good indirect index of the flavor intensity of onion products. Part of the unstable sulfenic acid also rearranges and decomposes to a rather large number of compounds in the classes of mercaptans, disulfides, trisulfides, and thiophenes. More extensive formation of these compounds and other derivatives also comprise the flavor substances which provide cooked onion flavors.

The flavor of garlic (*Allium sativum* L.) is formed by the same general type of mechanism that functions in onion, except that the precursor is *S*-(2-propenyl)-L-cysteine sulfoxide [70]. Diallyl thiosulfinate (allicin) (Figure 10.11) contributes to flavor of garlic, and an *S*-oxide lachyramator similar to that formed in onion is not formed. The thiosulfinate flavor compound of garlic decomposes and rearranges in much the same manner as indicated for the sulfenic acid of onion (Figure 10.10). This results in methyl, allyl, and diallyl disulfides and other principles in garlic oil and cooked garlic flavors.

10.3.2 Sulfur-Containing Volatiles in the *Cruciferae*

The *Cruciferae* family contains *Brassica* plants such as cabbage (*Brassica oleracea capitata* L.), brussels sprouts (*Brassica oleracea* var. *gemmifera* L.), turnips (*Brassica rapa* var. *rapa* L.), brown mustard (*Brassica juncea* Coss.), as well as water cress (*Nasturtium officinale* R. Br.), radishes (*Raphanus sativus* L.), and horseradish (*Armoracia lapathifolia* Gilib). As noted in the discussion about pungent compounds, the active pungent principles in the *Cruciferae* are also volatile and therefore contribute to characteristic aromas. Further, the pungent compounds in *Cruciferae* frequently cause pronounced irritation sensations, particularly in the nasal cavity, as well as lachrymatory effects. Flavor compounds in these plants are formed through enzymic processes in disrupted tissues and through cooking.

The fresh flavors of disrupted tissues are caused mainly by isothiocyanates resulting from the action of glucosinolases on thioglycoside precursors. The reaction shown in Figure 10.12 is

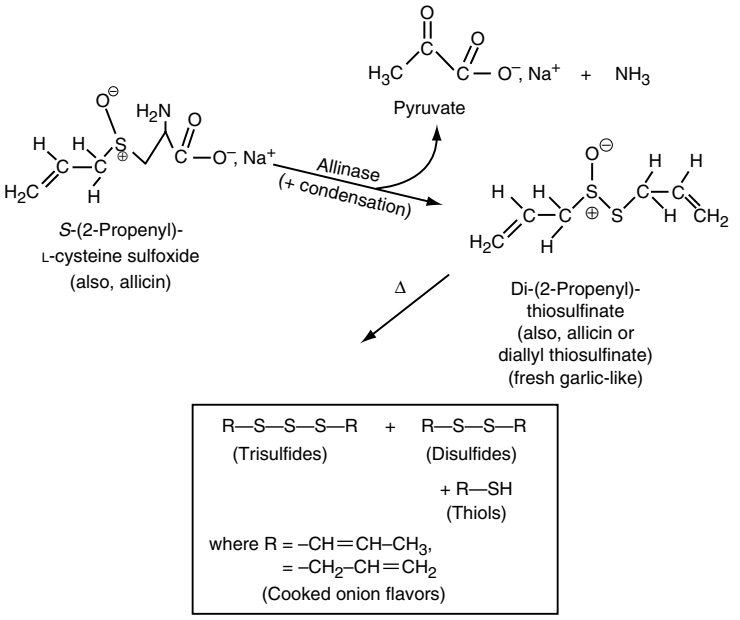


FIGURE 10.11 Reactions involved in the formation of garlic flavor compounds.

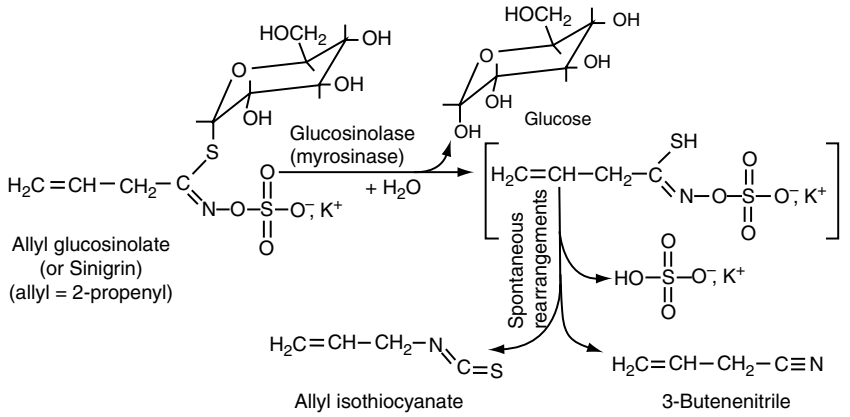
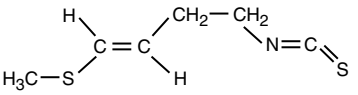


FIGURE 10.12 Reactions involved in the formation of *Cruciferae* flavors.

illustrative of the flavor-forming mechanism in fresh *Cruciferae*. Allyl isothiocyanate is the main source of pungency and aroma in horseradish and black mustard [24].

Several glucosinolates (*S*-glycosides, Chapter 12) occur in the *Cruciferae* [70], and each gives rise to characteristic flavors. The mild pungency of radishes is caused by the aroma compound, 4-methylthio-3-*t*-butenylisothiocyanate (XIX). In addition to the isothiocyanates, glucosinolates also yield thiocyanates and nitriles.



(XIX) 4-Methylthio-3-*t*-butenylisothiocyanate (radish)

Although not usually distinctly pungent, fresh cabbage and brussels sprouts contain the potential for both allyl isothiocyanate and allyl nitrile, and the concentration of each varies with the stage of growth, location in the edible part, and the severity of processing encountered. Processing at temperatures well above ambient (cooking and dehydrating) tends to destroy the isothiocyanates and enhance the amount of nitriles and other sulfur-containing degradation and rearrangement compounds. Several aromatic isothiocyanates occur in *Cruciferae*; for example, 2-phenylethyl isothiocyanate is one of the main aroma compounds of watercress. This compound also contributes a tingling pungent sensation which influences the flavors of salads containing watercress.

10.3.3 Unique Sulfur Compound in Shiitake Mushrooms

A novel C–S lyase flavor-related enzyme system occurs in Shiitake mushrooms (*Lentinus edodes*) that are prized in Japan and elsewhere for their unique desirable flavor. The precursor for the major flavor contributor, lentinic acid, is an *S*-substituted L-cysteine sulfoxide bound as a γ-glutamyl peptide [90]. The initial enzyme reaction in flavor development involves a γ-glutamyl transpeptidase, which releases a cysteine sulfoxide precursor (lentinic acid). Lentinic acid is then attacked by *S*-alkyl-L-cysteine sulfoxide lyase, which yields products that subsequently form lenthionine, the active flavor compound for Shiitake flavor (Figure 10.13). These reactions are initiated only after the tissue is disrupted, and the maximum flavor develops only after drying and rehydration or after holding freshly mascerated tissue for a period of time. Other polythiepanes in addition to lenthionine are formed, and contribute to the overall sulfurous flavor of flavor mushrooms [37,70].

10.3.4 Methoxy Alkyl Pyrazine Volatiles in Vegetables

Many fresh vegetables exhibit green-earthy aromas that contribute strongly to their recognition, and it has been found that the methoxy alkyl pyrazines are frequently responsible for this property [86]. Also, more recently, methoxy alkyl pyrazines have been associated with the flavors of certain varietal grape wines. These compounds have unusually potent and penetrating odors, and they provide strong identifying aromas. 2-Methoxy-3-isobutylpyrazine was the first of this class discovered, and it exhibits a powerful bell pepper aroma detectable at a threshold level of 0.002 ppb. Much of the aroma of raw potatoes, green peas, and pea pods is contributed by 2-methoxy-3-isopropylpyrazine, and

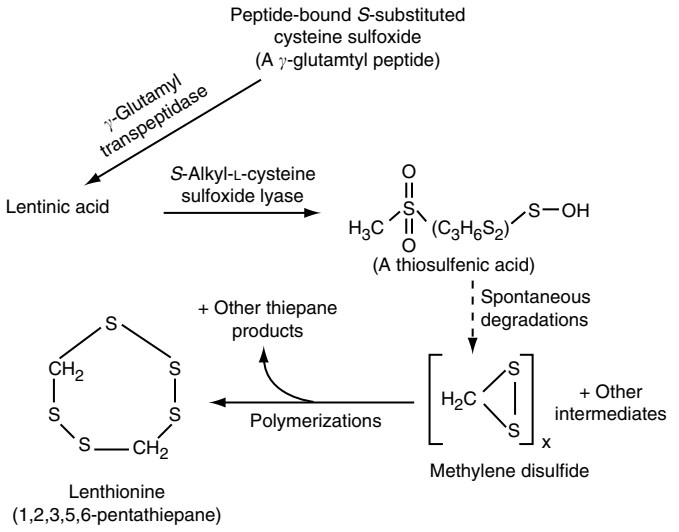


FIGURE 10.13 Reactions involved in the formation of lenthionine in Shiitake mushrooms.

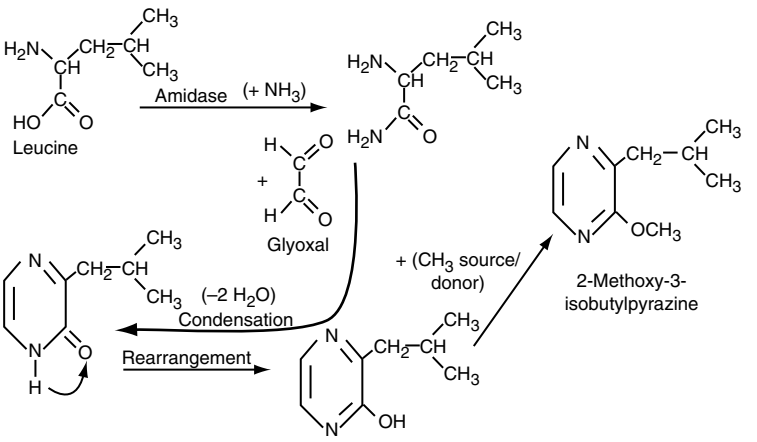


FIGURE 10.14 Proposed enzymic scheme for the formation of methoxy alkyl pyrazines.

2-methoxy-3-*sec*-butylpyrazine contributes to the aroma of raw red beet roots. These compounds arise biosynthetically in plants, and some strains of microorganisms (*Pseudomonas perolens* and *Pseudomonas tetrolens*) also actively produce these unique substances [53]. Branched-chain amino acids serve as precursors for methoxy alkyl pyrazine volatiles, and the mechanistic scheme shown in Figure 10.14 has been proposed.

10.3.5 Enzymically Derived Volatiles from Fatty Acids

Enzymically generated compounds derived from long-chain fatty acids play an extremely important role in the characteristic flavors of fruits and vegetables. In addition, these types of reactions can lead to important off-flavors, such as those associated with processed soybean proteins. Further information about these reactions can be found in the discussions about lipids (Chapter 4) and enzymes (Chapter 6).

10.3.5.1 Lipoxygenase-Derived Flavors in Plants

In plant tissues, enzyme-induced oxidative breakdown of unsaturated fatty acids occurs extensively, and this yields characteristic aromas associated with some ripening fruits and disrupted tissues [19]. In contrast to the random production of lipid-derived flavor compounds by purely autoxidizing systems, very distinctive flavors occur when the compounds produced are enzyme determined. The enzymic specificity for producing flavor compounds is illustrated in Figure 10.15, where 1-octene-3-one, *t*-2-*c*-6-nonadienal, and *t*-2-hexenal are formed from unsaturated fatty acids, and these compounds provide characterizing flavors to fresh mushrooms, cucumbers, and tomatoes, respectively. Site-specific peroxidations of liberated fatty acids are directed by specified lipoxygenases and subsequent lyase cleavage reactions. Upon cleavage of the fatty acid molecule, oxo-acids are also formed, but they do not appear to influence flavors.

Decompartmentalization of enzymes is required to initiate this and other reactions, and since successive reactions occur, overall flavors change with time. For example, the lipoxygenase-derived aldehydes and ketones are converted to corresponding alcohols (Figure 10.16) that usually have higher detection thresholds and heavier aromas than the parent carbonyl compounds. Additionally, *cis–trans* isomerases that are also present convert *cis*-3 bonds of aldehydes to *trans*-2 isomers (Figure 10.15), and these structural changes alter the aroma quality of the aldehydes. Generally, C_6 compounds yield green plant-like aromas-like fresh-cut grass, C_9 compounds smell like cucumbers and melons, and C_8 compounds smell like mushrooms or violet and geranium leaves [79]. The C_6

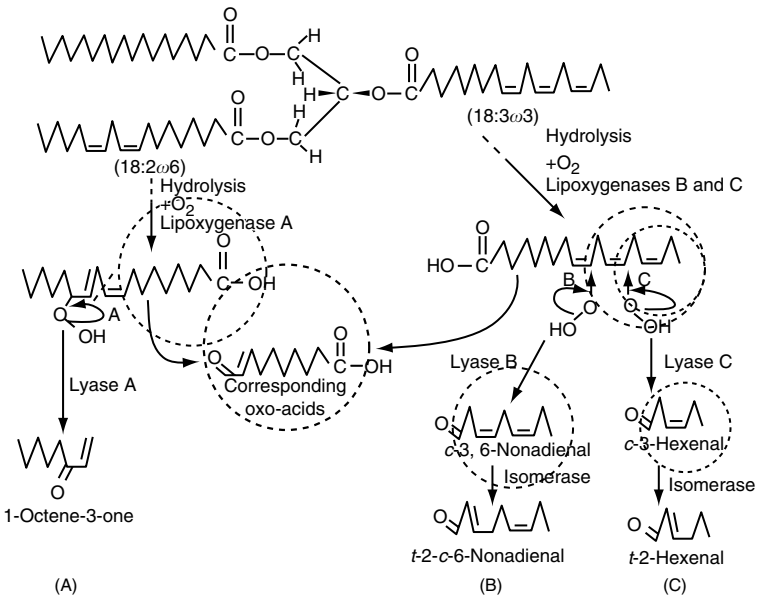


FIGURE 10.15 Formation of lipoxygenase-directed carbonyl compounds from unsaturated fatty acids: (A) important in fresh mushrooms; (B) important in cucumbers; and (C) important in fresh tomatoes.

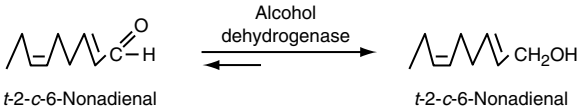


FIGURE 10.16 Conversion of an aldehyde to a corresponding alcohol resulting in a subtle flavor modification.

and C_9 compounds are primary alcohols and aldehydes; the C_8 compounds are secondary alcohols and ketones.

10.3.5.2 Volatiles from β-Oxidation of Long-Chain Fatty Acids

The development of pleasant, fruity aromas is associated with the ripening of pears, peaches, apricots, and other fruits, and these aromas are frequently dominated by medium-chain-length (C_6–C_{12}) volatiles derived from long-chain fatty acids by β-oxidation [80]. The formation of ethyl deca-2-*t*,4-*c*-dienoate by this means is illustrated in Figure 10.17. This ester is the impact or characterizing aroma compound in the Bartlett pear. Although not included in the figure, hydroxy acids (C_8–C_{12}) also may be formed as part of the overall enzymic process, and they readily cyclize to yield γ- and δ-lactones. Similar reactions occur during metabolism and biosynthesis of milk fat, and these reactions are discussed in more detail in Section 10.5. The C_8–C_{12} lactones possess distinct coconut- and peach-like aromas characteristic of these respective fruits.

10.3.6 Volatiles from Branched-Chain Amino Acids

Branched-chain amino acids serve as important flavor precursors for the biosynthesis of compounds associated with some ripening fruits. Bananas and apples are particularly good examples for this process because much of the ripe flavor of each is caused by volatiles from amino acids [80]. The

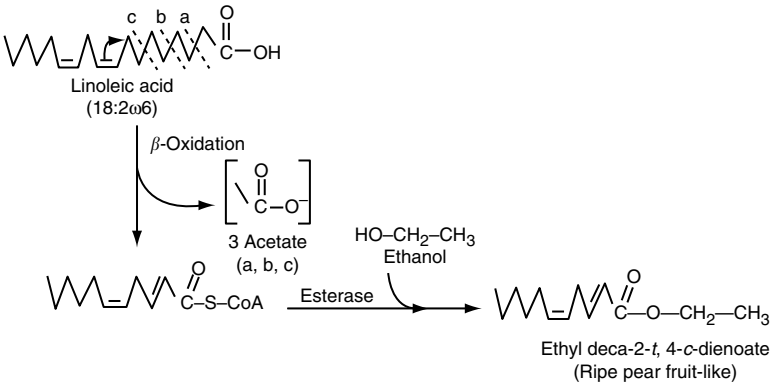


FIGURE 10.17 Formation of a key aroma substance in ripened pears through β-oxidation of linoleic acid followed by esterification.

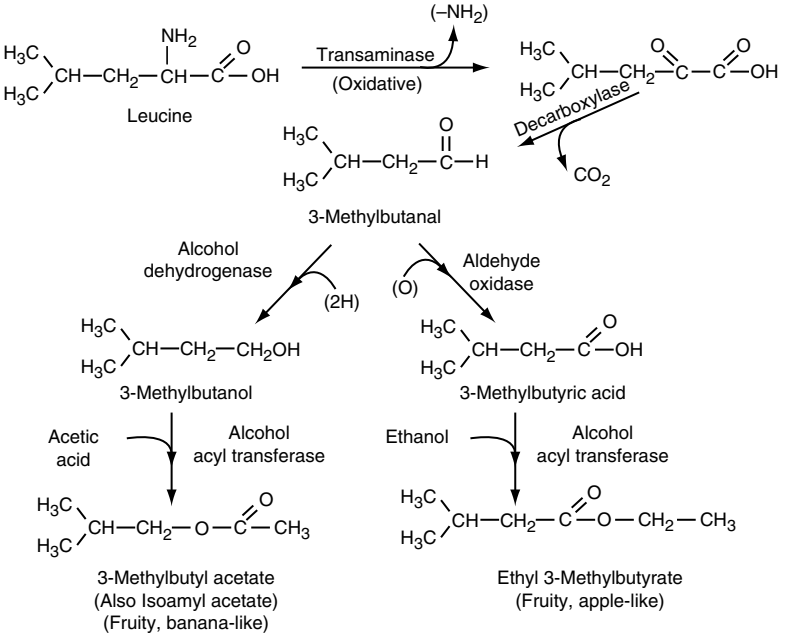


FIGURE 10.18 Enzymatic conversion of leucine to volatiles illustrating the aroma compounds formed from amino acids in ripening fruits.

initial reaction involved in flavor formation (Figure 10.18) is sometimes referred to as enzymic Strecker degradation because transamination and decarboxylation occur that is parallel to those occurring during nonenzymatic browning. Several microorganisms, including baker's yeast and malty flavor-producing strains of *Lactococcus lactis*, can also modify most of the amino acids in a fashion similar to that shown in Figure 10.18. Plants can also produce similar derivatives from amino acids other than leucine, and the occurrence of 2-phenethanol with a rose- or lilac-like aroma in blossoms is attributed to these reactions.

Although the aldehydes, alcohols, and acids from these reactions contribute directly to the flavors of ripening fruits, the esters are the dominant character-impact compounds. It has long been known that isoamyl acetate (3-methylbutyl acetate) is important in banana flavor, but other compounds are

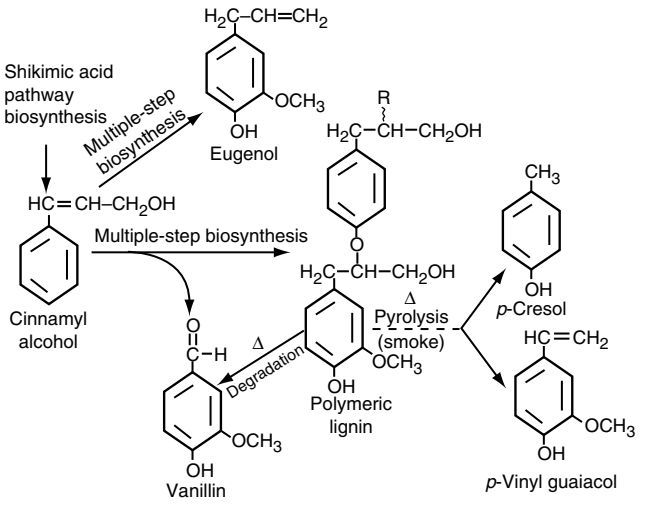


FIGURE 10.19 Some important flavor compounds variously derived from shikimic acid pathway precursors.

also required to give full banana flavors. Ethyl 2-methylbutyrate is even more apple-like than ethyl 3-methylbutyrate, and is the dominant note in the aroma of ripe, delicious variety apples.

10.3.7 Flavors Derived from the Shikimic Acid Pathway

In biosynthetic systems, the shikimic acid pathway provides the aromatic portion of compounds related to shikimic acid, and the pathway is best known in its role in the production of phenylalanine and other aromatic amino acids. In addition to flavor compounds derived from aromatic amino acids, the shikimic acid pathway provides other volatile compounds that are frequently associated with essential oils (Figure 10.19). It also provides the phenyl propanoid skeleton to lignin polymers that are the main structural elements of plants. As indicated in Figure 10.19, lignin yields many phenols during pyrolysis [87], and the characteristic aroma of smokes used in foods is largely caused by compounds developed from precursors in the shikimic acid pathway.

Also apparent from Figure 10.19 is that vanillin, the most important characterizing compound in vanilla extracts, can be obtained naturally via this pathway or as a lignin by-product during processing of wood pulp and paper. Vanillin is also biochemically synthesized in the vanilla bean where it is initially present largely as vanillin glucoside until the glycoside linkage is hydrolyzed during fermentation. The methoxylated aromatic rings of the pungent principles in ginger, pepper and chili peppers, discussed earlier in this chapter (Section 10.2.7), also contain many of the essential features of the compounds shown in Figure 10.19. Cinnamyl alcohol is an aroma constituent of cinnamon spice, and eugenol is the principal aroma and pungency element in cloves.

10.3.8 Volatile Terpenoids in Flavors

Because of the abundance of terpenes in plant materials used in the essential oil and perfumery industries, their importance in other plant-associated flavors is sometimes underestimated. They are largely responsible, however, for the flavors of citrus fruits and many seasonings and herbs. Volatile terpenes are present in low concentrations in several fruits, and are responsible for much of the flavor of raw carrot roots. Terpenes are biosynthesized through the isoprenoid (C_5) pathway (Figure 10.20), and monoterpenes contain 10 C atoms; the sesquiterpenes contain 15 C atoms. Sesquiterpenes are also important characterizing aroma compounds, and β-sinensal (XX) and nootkatone (XXI) serve

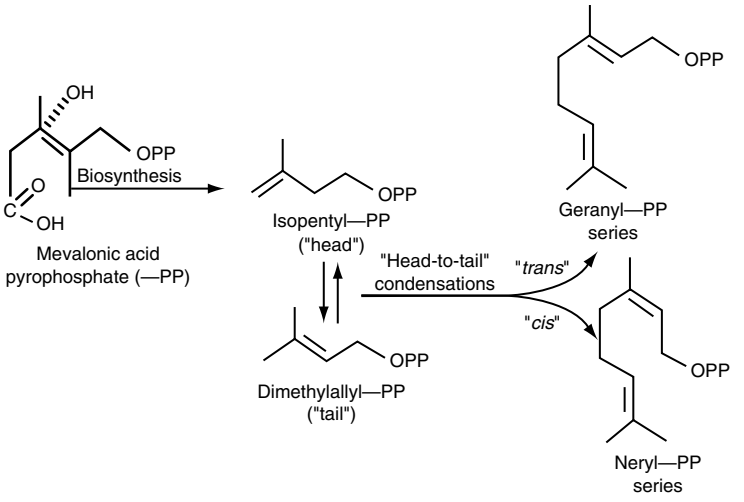
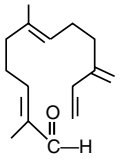
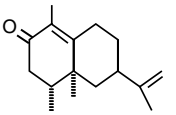


FIGURE 10.20 Generalized isoprenoid scheme for the biosynthesis of monoterpenes.

as good examples because they provide characterizing flavors to oranges and grapefruit, respectively. The diterpenes (C_{20}) are too large and nonvolatile to contribute directly to aromas.

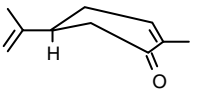


(XX) β-Sinesal (oranges)

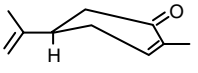


(XXI) Nootkatone (grapefruit)

Volatile terpenes frequently possess extremely strong character-impact aroma properties, and many can be identified easily by one experienced with natural product aromas. Optical isomers (i.e., structural mirror images) of terpenes, as well as optical isomers of other nonterpenoid compounds, can exhibit extremely different odor qualities [12,42,55]. In the case of terpenes, the carvones have been studied extensively from this perspective, and the aroma of *d*-carvone [4*S*-(+)-carvone] (XXII) has the characteristic aroma of caraway spice; *l*-carvone [4*R*-(−)-carvone] (XXIII) possesses a strong, characteristic spearmint aroma. Studies on such pairs of compounds are of special interest since they provide information on the fundamental process of olfaction and structure–activity relationships for molecules.



(XXII) 4*S*-(+)-Carvone (caraway like)



(XXII) 4*R*-(—)-Carvone (spearmint like)

10.3.9 Citrus Flavors

Citrus flavors are among the most popular fresh fruits as well as flavors for beverages, and most information about the flavor chemistry of natural citrus flavors stems from research on processed juices, peel essential oils, essence oils, and aqueous essences used to flavor juice products. Several classes of flavor components serve as major contributors to citrus flavors, including terpenes,

TABLE 10.2
Some Volatile Compounds Considered Important in Citrus Flavors

Orange	Mandarin	Grapefruit	Lemon
Ethanol	Ethanol	Ethanol	Neral
Octanol	Octanol	Decanal	Geranial
Nonanal	Decanal	Ethyl acetate	β-Pinene
Citral	α-Sinensal	Methyl butanoate	Geraniol
Ethyl butanoate	γ-Terpinene	Ethyl butanoate	Geranyl acetate
d-Limonene	β-Pinene	*d*-Limonene	Neryl acetate
α-Pinene	Thymol	Nootkatone	Bergamotene
	Methyl-*N*-methyl-anthranilate	1-*p*-Methene-8-thiol	Caryophyllene
			Carvyl ethyl ether
			Linalyl ethyl ether
			Fenchyl ethyl ether
			Methyl epijasmonate

Source: From Shaw, P. E. (1991). In *Volatile Compounds in Foods and Beverages* (H. Maarse, ed.), Marcel Dekker, New York, pp. 305–327.

aldehydes, esters, and alcohols, and large numbers of volatile compounds have been identified in the various extracts from each citrus fruit [71]. However, the important flavor compounds, including character-impact compounds, for citrus fruits generally are limited to relatively few, and those considered important to some major citrus fruits are shown in Table 10.2.

The flavors of orange and mandarin (tangerine is used interchangeably with mandarin in the United States) are delicate, and often are exchanged to various degrees in flavor applications. As can be noted from Table 10.2, relatively few aldehydes and terpenes are considered essential for these flavors, although a large number of other compounds are present. Both α- and β-sinensal (XX) are present in orange and mandarin flavors, and α-sinensal is considered especially important to providing a ripe orange-citrus flavor to mandarin flavors. Grapefruit contains two character-impact compounds, nootkatone (XXI) and 1-*p*-methene-8-thiol, which provide much of the easily recognized flavor to this fruit. Nootkatone is used extensively to provide artificial grapefruit flavor, and *p*-methene-8-thiol is one of apparently few sulfur compounds that are influential in citrus flavors.

Lemon flavor requires contributions from a large number of important compounds, and lemon flavors benefit from the presence of several terpene ethers. Similarly, the number of essential compounds in lime flavor is large, and two types of lime oil are commonly used. The major lime oil in commerce is distilled Mexican lime oil that possesses a harsh, strong lime flavor that is popular in lemon–lime and cola soft drinks. Cold-pressed Persian lime oil and centrifuged Mexican lime oils are becoming more popular because they possess a more natural flavor. The less rigorous processing involved in cold-pressing and centrifugation, compared to distillation, results in the survival of some of the more sensitive, but important fresh lime flavor compounds. For example, citral, which has a desirable fresh-like aroma is degraded under acidic distillation conditions to *p*-cymene and α–*p*-dimethylstyrene that have coarse flavors, and these conversions lead to the more harsh flavors of distilled lime oil [71].

Citrus essential oils, or flavor extracts containing terpenes, can be separated into nonoxygenated (hydrocarbon) and oxygenated fractions using silicic acid chromatography and nonpolar and polar solvent elutions, respectively. Terpeneless orange oil, for example, contains principally the oxygenated terpenes, aldehydes and alcohols that have been recovered from orange oils. Because of their higher flavor quality, oxygenated terpene fractions are frequently more desired in flavors than nonoxygenated fractions.

10.3.10 Flavors of Herbs and Spices

Although some variations occur between industrial and the various domestic and international regulatory agencies regarding definitions, spices and herbs are known and regulated as spices and condiments that are natural vegetable products used for flavoring, seasoning, and imparting aroma to foods. The U.S. Food and Drug Administration excludes alliaceous products, such as onions and garlic, from classification as spices, but international and industrial spice classifications commonly include these materials. The term condiments has been retained in regulatory definitions, and is defined as something to enhance the flavor of food, or a pungent seasoning. It has been argued that the term should be dropped from usage, but some flavor retention of the term for certain situations. However, the term generally does not provide a basis for inclusion in classification schemes for aromatic plant materials.

In some botanically based classification schemes, culinary herbs are separated from spices, and include such aromatic, soft-stemmed plants as basil, marjoram, mints, oregano, rosemary, and thyme as well as aromatic shrub (sage) and tree (laurel) leaves. In such classifications, spices comprise all other aromatic plant materials used in the flavoring or seasoning of foods. Such spices generally lack chlorophyll, and include rhizomes or roots (ginger), barks (cinnamon), flower buds (cloves), fruits (dill, pepper), and seeds (nutmeg, mustard).

Spices and herbs have been used since antiquity for adding savoriness, tanginess, and zestiness as well as characterizing flavors to foods and beverages. Some also have been used extensively in perfumery and for medicinal purposes, and many exhibit antioxidant or microbial inhibitory effects. While many herbs and spices exist throughout the world, some of which are used for perfumery and herbal medicines, only about 70 are officially recognized as useful ingredients for foods. However, the flavor characteristics often vary for a given spice depending on location of growth and genetic variations, thus this group provides a wide range of flavors for foods. Here, only those spices and herbs that are commonly used in the food industry for flavoring are considered.

Spices are generally derived from tropical plants while herbs are generally derived from subtropical or temperate plants. Spices also generally contain high concentrations of phenylpropanoids from the shikimic acid pathway (e.g., eugenol in cloves; see Figure 10.19), herbs generally contain higher concentrations of *p*-menthanoids from terpene biosynthesis (e.g., menthol in peppermint; see XVII)

Typically, spices and herbs contain a large number of volatile compounds, but in most instances certain compounds, either abundant or minor volatile constituents, provide character-impact aromas and flavors to the material. Important flavor compounds found in the principal herbs and spices used by the food industry are summarized in Tables 10.3 and 10.4 [12,64], respectively. Successful applications of herbs and spices in foods and beverages require either a personal knowledge of the materials or knowledge of the dominant and subtle flavor notes provided by flavor chemicals. Evaluation of the important flavor compounds listed in Tables 10.3 and 10.4 for spices and herbs shows which spices and herbs provide related flavors, and also indicate the type of flavors to be expected from their usage. Still, it must be remembered that a large number of compounds of lesser influence are present and also contribute to the unique flavors of herbs and spices.

10.4 FLAVORS FROM LACTIC ACID–ETHANOL FERMENTATIONS

Involvement of microorganisms in flavor production is extensive, but often their specific or definitive role in the flavor chemistry of fermentations is not well-known or the flavor compounds do not have great character impact. Much attention has been given to cheese flavor, but apart from the distinctive flavor properties given by methyl ketones and secondary alcohols to blue-mold cheeses and the moderate flavor properties given by certain sulfur compounds to surface-ripened cheeses, microbially derived flavor compounds cannot be classified in the character-impact category. Similarly, yeast fermentations, carried out extensively for beer, wines, spirits, and yeast-leavened breads,

TABLE 10.3
Important Flavor Compounds Found in Some Culinary Herbs Commonly Used for Flavoring in the Food Industry

Herbs	Plant Part	Important Flavor Compounds
Basil, sweet	Leaves	Methylchavicol, linalool, methyl eugenol
Bay laurel	Leaves	1,8-Cineole
Marjoram	Leaves, flowers	*c* and *t*-Sabinene hydrates, terpinen-4-ol
Oregano	Leaves, flowers	Carvacrol, thymol
Origanum	Leaves	Thymol, carvacrol
Rosemary	Leaves	Verbenone, 1,8-cineole, camphor, linalool
Sage, clary	Leaves	Salvial-4(14)-en-1-one, linalool
Sage, Dalmation	Leaves	Thujone, 1,8-cineole, camphor
Sage, Spanish	Leaves	*c* and *t*-Sabinyl acetate, 1,8-cineole, camphor
Savory	Leaves	Carvacrol
Tarragon	Leaves	Methyl chavicol, anethole
Thyme	Leaves	Thymol, carvacrol
Peppermint	Leaves	*l*-Menthol, menthone, menthofuran
Spearmint	Leaves	*l*-Carvone, carvone derivatives

Source: From Boelens, M. H., et al. (1993). *Perfumer Flavorist* 18:1–16 and Richard, H. M. J. (1991). In *Volatile Compounds in Foods and Beverages* (H. Maarse, ed.), Marcel Dekker, New York, pp. 411–447.

do not appear to yield strong, distinctive character-impact flavor compounds. Ethanol in alcoholic beverages, however, should be considered as having character impact.

The primary fermentation products of heterofermentative lactic acid bacteria (e.g., *Leuconostoc citrovorum*) are summarized in Figure 10.21, and the combination of acetic acid, diacetyl, and acetaldehyde provides much of the characteristic aroma of cultured butter and buttermilk. Homofermentative lactic acid bacteria (e.g., *Lactococcus lactis, Streptococcus thermophilus*) produce only lactic acid, acetaldehyde, and ethanol in milk cultures. Acetaldehyde is the character-impact compound found in yoghurt, a product generally prepared by a homofermentative process. Diacetyl is the character-impact compound in most mixed-strain lactic fermentations, and has become universally known as a dairy or butter-type flavorant. Acetoin, although essentially odorless, can undergo oxidation to diacetyl. Lactic acid is nonvolatile and contributes only sourness to cultured or fermented dairy products.

Viewed in general terms, lactic acid bacteria produce very little ethanol (parts per million levels) and they use pyruvate as the principal final H acceptor in metabolism. On the other hand, yeast, produce ethanol as a major end product of metabolism. Malty strains of *Lactococcus lactis* and all brewer's yeasts (*Saccharomyces cerevisiae, Saccharomyces carlsbergensis)* also actively convert amino acids to volatile compounds through transaminations and decarboxylations. In the example shown in Figure 10.22, phenylalanine yields a series of aromatic volatile compounds that generally are perceived as floral or rose like. Aged Cheddar cheese containing excessive phenethylalcohol production is often considered defective in part because cheeses with the rose-like flavor in the past have been associated with uncontrolled lactic fermentations resulting from the use of incompletely cleaned dairy equipment.

The reactions shown for phenylalanine are analogous to those discussed for branched-chain amino acids in Section 10.3.6. However, these microorganisms tend to produce mainly the reduced forms of the derivatives (alcohols), although some oxidized compounds (aldehydes and acids) also appear. Wine and beer flavors, which can be ascribed directly to fermentations, involve complex

TABLE 10.4
Important Flavor Compounds Found in Some Spices Commonly Used for Flavoring in the Food Industry

Spice	Plant Part	Important Flavor Compounds
Allspice (pimento)	Berry, leaves	Eugenol, β-caryophyllene
Anise	Fruit	(*E*)-Anethole, methyl chavicol
Capsicum peppers	Fruit	Capsaicin, dihydrocapsaicin
Caraway	Fruit	*d*-Carvone, carvone derivatives
Cardamom	Fruit	α-Terpinylacetate, 1,8-cineole, linalool
Cinnamon, cassia	Bark, leaves	Cinnamaldehyde, eugenol
Clove	Flower bud	Eugenol, eugenylacetate
Coriander	Fruit	*d*-Linalool, C_{10}–C_{14} 2-alkenals
Cumin	Fruit	Cuminaldehyde, *p*-1,3-menthadienal
Dill	Fruit, leaves	Anethofuran, *d*-carvone
Fennel	Fennel	(*E*)-Anethole, fenchone
Ginger	Rhizome	Gingerol, shogaol, neral, geranial
Mace	Aril	α-Pinene, sabinene, 1-terpenin-4-ol
Mustard	Seed	Allyl isothiocyanate
Nutmeg	Seed	Sabinene, α-pinene, myristicin
Parsley	Leaves, seed	Apiol
Pepper	Fruit	Piperine, δ-3-carene, β-carophyllene
Saffron	Stigma	Safranal
Turmeric	Rhizome	Tumerone, zingeriberene, 1,8-cineole
Vanilla	Fruit, seed	Vanillin, *p*-OH-benzyl methyl ether

Source: From Boelens, M. H., et al. (1993). *Perfumer Flavorist* 18:1–16 and Richard, H. M. J. (1991). In *Volatile Compounds in Foods and Beverages* (H. Maarse, ed.), Marcel Dekker, New York, pp. 411–447.

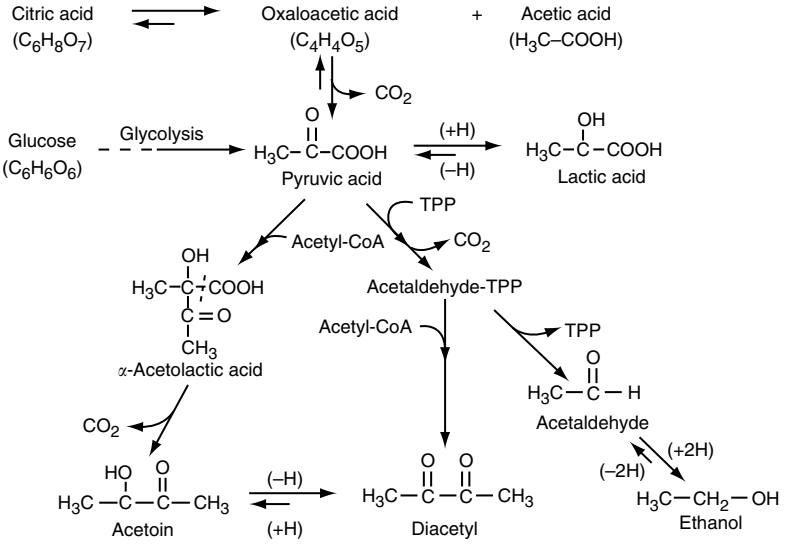


FIGURE 10.21 Formation of the principal flavor-related fermentation products in heterofermentative lactic acid bacterial metabolism (TPP = thiamin pyrophosphate).

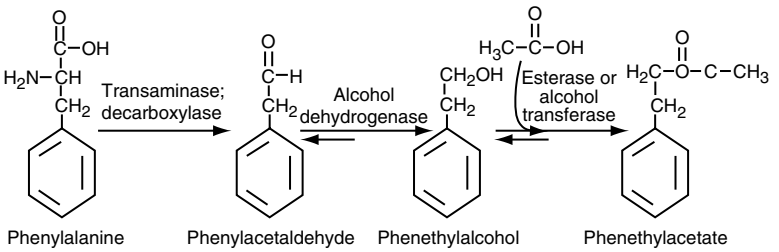


FIGURE 10.22 Enzymatic formation of volatiles from amino acids by microorganisms using phenylalanine as a model precursor compound.

mixtures of these volatiles and interaction products of these compounds with ethanol, such as mixed esters and acetals. These mixtures give rise to familiar yeasty and fruity flavors associated with fermented beverages.

10.5 FLAVOR VOLATILES FROM FATS AND OILS

Fats and oils are notorious for their role in the development of off-flavors through autoxidation, and the chemistry of lipid-derived flavors has been well-summarized [26,38]. Aldehydes and ketones are the main volatiles from autoxidation, and these compounds can cause painty, fatty, metallic, papery, and candle-like flavors in foods when their concentrations are sufficiently high. However, many of the desirable flavors of cooked and processed foods derive from modest concentrations of these compounds. Details of lipid autoxidation mechanisms and other degradations of lipids are largely presented in Chapter 4.

10.5.1 Flavors from the Hydrolysis of Fats and Oils

Hydrolysis of plant acylglycerols and animal depot fats leads mainly to the formation of potentially soapy-tasting fatty acids. Milk fat, on the other hand, serves as rich source of volatile flavor compounds that are influential in the flavor of dairy products and foods prepared with milk fat or butter. The classes of volatiles obtained by hydrolysis of milk fat are shown in Figure 10.23, with specific compounds selected to illustrate each class. The even C-numbered, short-chain fatty acids (C_4–C_{12}) are extremely important in the flavor of cheese and other dairy products, with butyric acid being the most potent and influential of the group. Hydrolysis of hydroxy-fatty acids leads to the formation of lactones which provide desirable coconut- or peach-like flavor notes to baked goods, but cause staling in stored, sterile concentrated milks. Methyl ketones are produced from β-ketoacids by heating after hydrolysis, and they contribute to the flavor of dairy products in much the same manner as the lactones. In blue-mold cheeses, however, methyl ketones are much more abundantly produced by the metabolic activities of *Penicillium roqueforti* on fatty acids than by the conversion of the ketoacids that are bound in acylglycerols.

Although hydrolysis of fats other than milk fat in general does not yield distinct flavors as noted above, animal fats are believed to be inextricably involved in the species-related flavors of meats. The role of lipids in species-related aspects of meat flavors is discussed in the next section on muscle foods and milk (Section 10.6).

10.5.2 Distinctive Flavors from Long-Chain Polyunsaturated Fatty Acids

Many consider cooking with animal fats (i.e., lard, beef tallow, etc.) to yield foods with significantly different and better flavors than those cooked with any of the current plant-derived fats or oils.

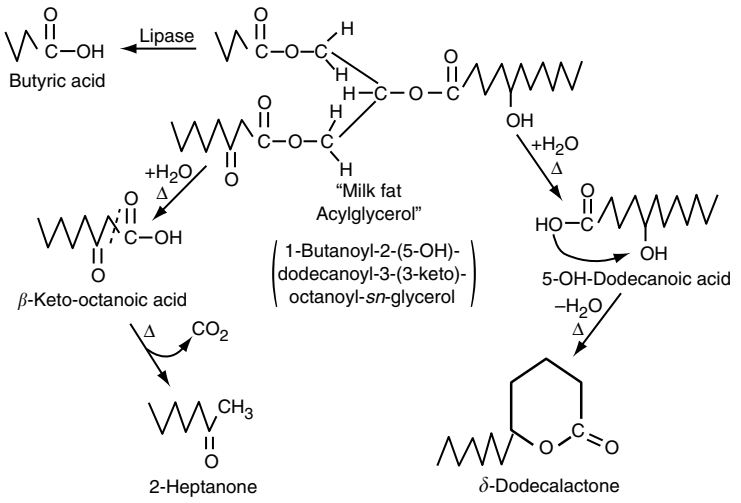


FIGURE 10.23 Formation of influential volatile flavor compounds from milk fat obtained through hydrolytic cleavage of acylglycerols.

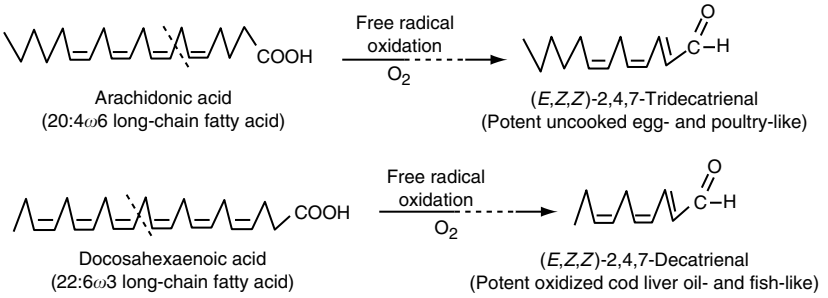


FIGURE 10.24 Formation of distinctive volatile flavor compounds from long-chain polyunsaturated fatty acids.

The reasons considered responsible for the different flavors have included differing bulk fatty acid compositions that yield different oxidation flavor compound profiles and differing minor components that are dissolved in the respective fats. However, the chemical basis for these differences is still debated.

Terrestrial plant-derived food fats and oils currently contain only polyunsaturated fatty acids with 18 C (mainly 18:2:ω6 and 18:3:ω3) or less. However, while animal-derived fats and products contain bulk polyunsaturated fatty acids similar to plant fats and oils, they also contain notable amounts of the long-chain polyunsaturated fatty acid, arachidonic acid (20:4:ω6). Recent research has clarified the primary flavorful oxidation products derived from arachidonic acid [10], and these findings provide some insights into the key flavor chemistry differences between plant and animal fats. Oxidizing pure arachidonic acid emanates distinctive, sometimes unpleasant, uncooked poultry- and animal-like aromas, and the aromas are substantially caused by (E,Z,Z)-2,4,7-tridecatrienal that in the pure form exhibits potent uncooked egg- and poultry-like aromas (Figure 10.24). Since this unsaturated oxidation product of arachidonic acid is so potent and distinct, it is likely the characterizing or character-impact compound responsible for the casual observation that "many white meats taste a lot like chicken."

Animal fats also contain ω3 long-chain polyunsaturated fatty acids although in notably less abundance than arachidonic acid, but fish oils contain relatively large amounts of these fatty acids.

Included are docosahexaenoic acid (22:6ω3; DHA) and eicosapentaenoic acid (EPA; 205:ω3), and oxidation of either yields, among other products, (E,Z,Z)-2,4,7-decatrienal which possesses potent oxidized cod liver oil-like or stale fish-like flavors (Figure 10.24). In excessive concentrations, (E,Z,Z)-2,4,7-decatrienal causes extremely undesirable fishy flavors, but at acceptable levels, it imparts desirable characterizing or character-impact flavor properties to fish and seafoods. Thus, the products of ω3 long-chain polyunsaturated fatty acid oxidations provide a chemical basis for differentiating between the flavors of aquatic and terrestrial animal foods. However, it is also likely that the ω3 long-chain polyunsaturated fatty acids are also involved in the flavor differences resulting from plant and animal fats and oils. Notably, with commercial algal culturing and genetic engineering advances that have occurred recently, it is certain that the traditional differentiating flavor features between plant and animal fats and oils rapidly will become obscured.

10.6 FLAVOR VOLATILES IN MUSCLE FOODS AND MILK

The flavors of meats have attracted much attention, but in spite of considerable research, knowledge about the flavor compounds causing strong character impacts for meats of various species remains somewhat obscure [17,68]. Nevertheless, the concentrated research efforts on meat flavors have produced a wealth of information about compounds which contribute to cooked meat flavors. The somewhat distinctive flavor qualities of meat flavor compounds that are not species-related are very valuable to the food and flavor industry, but chemical definitions of lightly cooked and species-related flavors are still eagerly sought. Some details of the chemistry relating to the flavors of well-cooked meat are discussed in Section 10.7

10.6.1 Species-Related Flavors of Meats and Milk from Ruminants

The characterizing flavors of at least some meats are inextricably associated with the lipid fraction. Although long a subject of debate, significant advances in defining species-related flavors were initiated by Wong and coworkers [88] in relation to lamb and mutton flavors. These workers showed that a characteristic sweat-like flavor of mutton was closely associated with some volatile, medium-chain-length fatty acids of which some methyl-branched members are highly significant. The formation of one of the most important branched-chain fatty acids in lamb and mutton, 4-methyloctanoic acid, is shown in Figure 10.25.

Ruminal fermentations yield acetate, propionate, and butyrate, but most fatty acids are biosynthesized from acetate, which yields straight or nonbranched chains. However, some methyl branching occurs routinely because of the presence of propionate, but when dietary and other factors enhance the propionate concentrations in the rumen, greater methyl branching occurs [73]. Several medium-chain, methyl-branched fatty acids are important contributors to species-related flavors, including 4-methylnonanoic acid in lamb and mutton flavors. Additionally, 4-ethyloctanoic acid (threshold = 1.8 ppb in water) is particularly important for conveying very distinctive goat-like flavors to both meats and milk products, and it is formed when the initial primer fatty acid is butyric acid (butyrl-CoA) rather than propionic acid (propionyl-CoA , and it is coupled with a butyrl-enzyme moiety as shown in Figure 10.25 [29–31].

Several alkylphenols (methylphenol isomers, ethylphenol isomers, *n*-propyl isomers, isopropylphenol isomers, and methyl-isopropylphenol isomers) contribute very characteristic beef- and sheep-like species-related flavors to meats and milks [31,32,48]. Additionally, some alkylphenols, especially *m*-substituted members, exhibit kokumi-like flavor properties (Section 10.2.6). Alkylphenols are present as free (flavorful) and conjugate-bound (flavorless) substances in meat and milk, and are initially derived via rumen fermentation conversions of shikimic acid pathway biochemicals found in forages (Figure 10.26). Sulfate, phosphate, and glucuronide conjugates of alkylphenols are

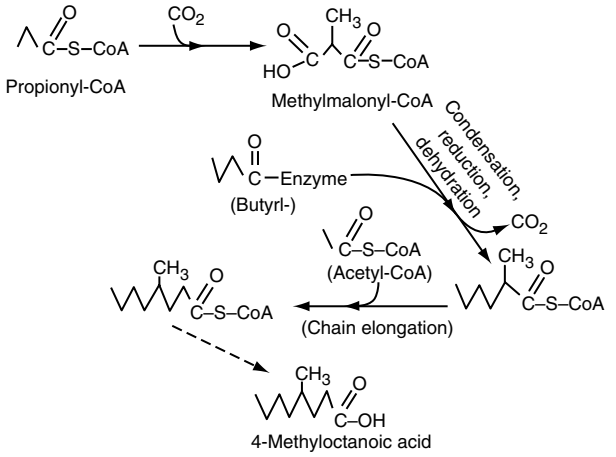


FIGURE 10.25 Reactions showing ruminant biosynthesis of methyl-branched, medium-chain fatty acids.

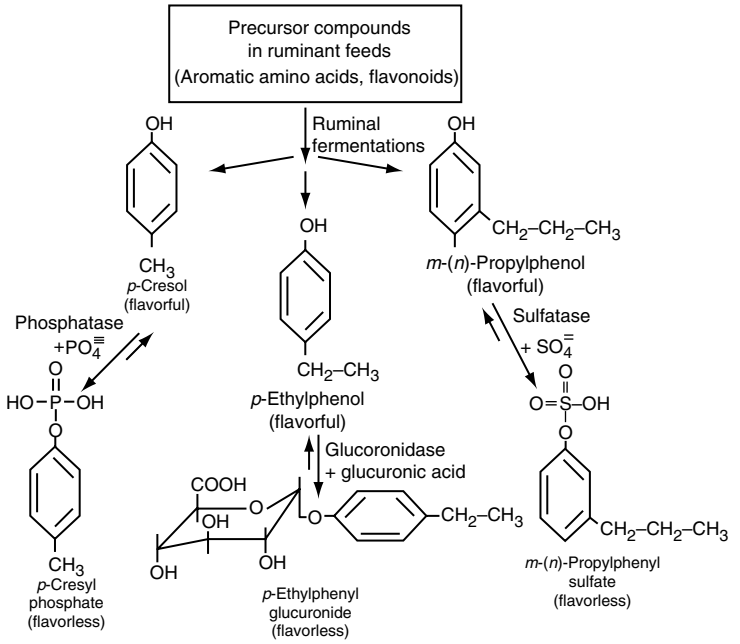


FIGURE 10.26 Formation of alkylphenols from feeds by ruminants.

formed *in vivo* in ruminants to improve the water solubility of alkylphenols to enhance their elimination in the urine. Subsequently, both enzymic and thermal hydrolysis may release alkylphenols from conjugates, and thereby enhance flavor development during fermentations and cooking of meat and dairy products.

10.6.2 Species-Related Flavors of Meats from Nonruminants

Species-related aspects of the flavor chemistry of nonruminant meats appear to remain somewhat incomplete. The distinct pork-like or piggy flavor, noticeable in lard or cracklings and in some

pork, is caused in part by *p*-cresol and isovaleric acid that are produced from microbial conversions of corresponding amino acids in the lower gut of swine [29,31]. Similar formation of indole and skatole from tryptophan may also intensify unpleasant piggy flavors in pork. Excessive levels of (*E*, *Z*, *Z*)-2,4,7-tridecatrienal from arachidonic acid oxidation (Section 10.5.2) also accentuates piggy flavors, but acceptable concentrations (see Section 10.1.3) of this compound contribute desirably to pork flavors. Also, studies have shown that the γ-C_5, C_9, and C_{12} lactones are reasonably abundant in pork [17], and these compounds may contribute some of the sweet-like flavor of swine meat.

Much interest has centered on the aroma compounds responsible for the swine sex odor that causes serious off-flavors in pork. Two compounds that contribute much to the off-flavor are 5-α-androst-16-en-3-one which has a urinous aroma and 5-α-androst-16-en-3α-ol which has a musk-like aroma (Figure 10.27; [25]). The swine sex odor compounds are mainly associated with males, but may occur in castrated males and in females. These steroid compounds are particularly offensive to some individuals, especially women, and yet others are genetically odor-blind to them. Since the compounds responsible for the swine sex odor have only been found to cause off-flavors in pork, they can be regarded as species-related flavor compounds for swine.

The distinctive flavors of poultry have also been the subject of many studies, and lipid oxidation yields the character-impact compounds for chicken. Early research [33] implicated the carbonyls, *c*-4-decenal, *t*-2-*c*-5-undecadienal, and *t*-2-*c*-4-*t*-7-tridecatrienal in the characteristic flavor of stewed chicken, and they were believed to be derived from linoleic and arachidonic acids. However, a recent detailed reevaluation [10] of the volatile products from the autoxidation of arachidonic acid (also see Section 10.5.2) has shown that (*E*, *Z*, *Z*)-2,4,7-tridecatrienal from the reaction is an extremely potent poultry-like substance, and thus is a character-impact compound for chicken and other white meat flavors. Other factors, such as cooking method, also influence the characteristics of cooked poultry flavors. One likely influential factor is that chickens accumulate α-tocopherol (an antioxidant), but turkeys do not, and during cooking, especially roasting, carbonyls are formed to a much greater extent in turkey than in chicken.

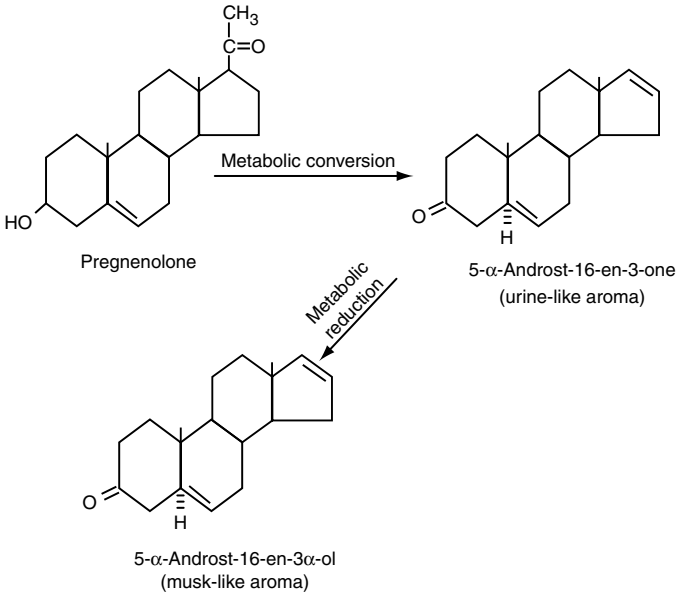


FIGURE 10.27 Formation of steroid compounds responsible for urine- and musk-like aromas associated with the swine sex odor defect of pork.

10.6.3 Volatiles in Fish and Seafood Flavors

Flavors in seafoods cover a somewhat broader range of flavor qualities than those occurring in other muscle foods. The broad range of animals involved (finfish, shellfish, and crustaceans), and the variable flavor and aroma qualities related to freshness each account for the different flavors that are encountered. Because the fresh flavors and aromas of seafoods frequently have been greatly diminished or lost from fresh-stored, frozen, and processed products available through commercial channels, many consumers associate secondarily developed stale and fishy flavors with all fish and seafoods. However, very fresh seafoods exhibit delicate aromas and flavors quite different from those usually evident in "commercially fresh" seafoods. One contributing factor is the accumulation and subsequent decline of the umami substance, inosine-5′-monophosphate (Section 10.2.5), in tissues that cause dramatic time-related changes in the flavors of refrigerated fish and seafoods.

A group of lipoxygenase-derived C_6, C_8, and C_9 aldehydes, ketones and alcohols initially provide the characterizing and pleasant aroma and flavor of fresh fish [47]. Overall the flavor compounds produced in fish and seafoods are similar to those produced by plant lipoxygenases (Section 10.3.5). However, the lipoxygenases found in fish and seafoods perform enzymic oxidations related to leucotriene synthesis, and flavor compound production is a by-product of those reactions. For the example shown in Figure 10.28, peroxidation followed by disproportionation reactions apparently lead first to the alcohol, and then to the corresponding carbonyl [75]. Collectively, these compounds provide melon-like, green plant-like notes to fresh fish aromas, and contribute to the delicate flavors of very fresh cooked fish, either directly or as reactants that lead to new flavors during cooking.

The flavors of crustaceans and mollusks rely particularly heavily on nonvolatile taste substances in addition to contributions from volatiles. For example, the taste of cooked snow crab meat has been claimed to be largely duplicated with a mixture of 12 amino acids, nucleotides, and salt ions [41]. Good imitation crab flavors can be prepared from the taste substances just mentioned, along with some contributions from carbonyls and trimethylamine. Trimethylamine has long been associated with fish- and crab-like aromas, and it alone exhibits an ammoniacal, fishy aroma. Trimethylamine and dimethylamine are produced through microbial or endogenous enzymic degradation of trimethylamine oxide (Figure 10.29) that is found in significant quantities only in saltwater species

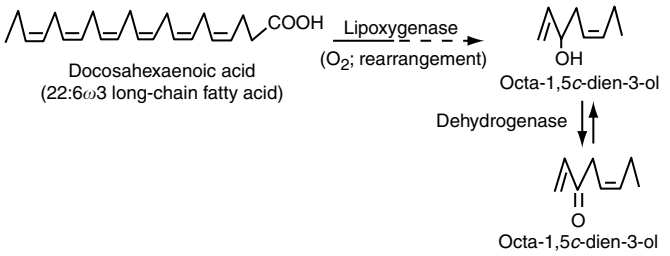


FIGURE 10.28 Enzymatic formation of influential volatiles in fresh fish aroma from long-chain $\omega 3$ unsaturated fatty acid.

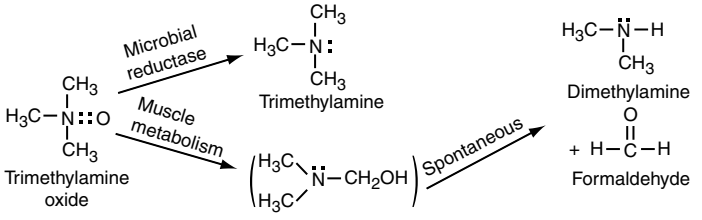


FIGURE 10.29 Formation of principal volatile amines in muscle tissues of saltwater fish species.

of fish and seafoods. Trimethylamine oxide serves as part of the osmolyte system in marine or saltwater fish species [36]. Since very fresh fish contain essentially no trimethylamine, this compound modifies and contributes to the aroma of staling fresh fish in which it enhances fishhouse-type aromas. Dimethylamine formation is most often associated with staling that occurs during frozen storage. The formaldehyde produced concurrently with dimethylamine is believed to facilitate protein crosslinking, and thereby contributes to the toughening of fish muscle during frozen storage.

Other aromas and flavors associated with fish products are often characterized by such terms as "stale or oxidized fish" and "cod liver oil like," and these are caused by certain carbonyl compounds that develop from the autoxidation of $\omega 3$ long-chain polyunsaturated fatty acids (Section 10.5.2). Of these, (E, Z, Z)-2,4,7-decatrienal (cf. Figure 10.24) provides an especially important character-impact fishy flavor quality, and *c*-4-heptenal potentiates its fishy character [47].

Some important characterizing flavors in fish and seafoods are derived from the environment, principally through the natural food chain for the harvested species. For example, dimethyl sulfide provides a characterizing top-note aroma to cooked clams and oysters, and it arises principally from the thermal degradation of dimethyl-β-propiothetin (Figure 10.30) present in ingested marine microflora [56] whose presence in the habitat is dependent on the environmental conditions.

The flavors of aquacultured shrimp and salmon are affected by their diets that differ substantially from those encountered by free-roaming wild counterparts [56]. One of the key differences in the corresponding flavors of these animals is caused by a general lack of bromophenols in aquaculture diets compared to those consumed by wild-capture counterparts. Bromophenols are produced metabolically (Figure 10.31) by a variety of lower-form marine or saltwater organisms, and they are passed up through the food chain where they provide character-impact flavors to both fish and other seafoods [13]. One of the most notable flavor effects of bromophenols is encountered in wild-capture shrimp where the flavor may vary from subtle sea like to distinctly iodine like. On the other hand,

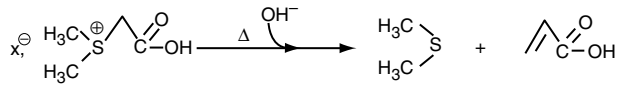


FIGURE 10.30 Formation of dimethyl sulfide in seafoods.

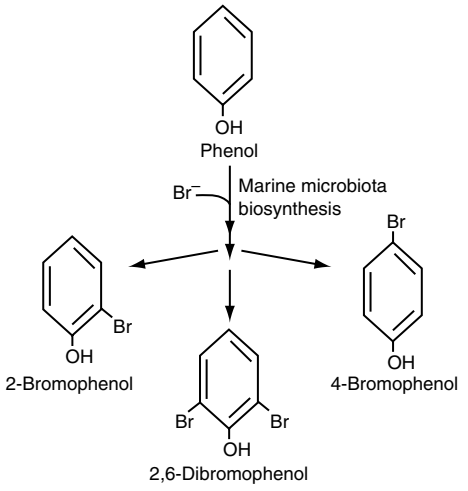


FIGURE 10.31 Formation of bromophenols in marine seafoods.

the very low levels of bromophenols that occur in aquacultured shrimp result in indistinct or bland flavors that lack traditional marine- or sea-like flavor notes.

10.7 DEVELOPMENT OF PROCESS OR REACTION FLAVOR VOLATILES

Many flavor compounds found in cooked or processed foods occur as the result of reactions common to all types of foods regardless of whether they are of animal, plant or microbial derivation. These reactions take place when suitable reactants are present and appropriate conditions (heat, pH, light) exist. Process or reaction flavors are discussed separately in this section because of their broad importance to all foods, and because they comprise a large volume of natural flavor concentrates that are used widely in foods, especially when meat or savory flavors are desired. Related information can be found in discussions dealing with carbohydrates (Chapter 3), lipids (Chapter 4), and vitamins (Chapter 7).

10.7.1 Thermally Induced Process Flavors

Traditionally, these flavors have been broadly viewed as products from browning reactions because of early discoveries showing the role of reducing sugars and amino compounds in the induction of a process that ultimately leads to the formation of brown pigments (see Chapters 3 and 4 for details of Maillard browning reactions). Although browning reactions are almost always involved in the development of process flavors in foods, the interactions between products of the browning reaction and other food constituents are also important and extensive. By taking a broad approach to discussions of thermally induced flavors, the aforementioned interactions, as well as reactions that occur following heat treatment, can be appropriately considered.

Although many of the compounds associated with process flavors possess potent and pleasant aromas, relatively few of these compounds seem to provide truly distinguishing character-impact flavor effects. Instead they often contribute general nutty, meaty, roasted, toasted, burnt, floral, plant, or caramel odors. Some process flavor compounds are acyclic, but many are heterocyclic, with nitrogen, sulfur, or oxygen substituents common (Figure 10.32). These process flavor compounds occur in many foods and beverages, such as roasted meats, boiled meats, coffee, roasted nuts, beer, bread, crackers, snack foods, cocoa, and most other processed foods. The distribution of individual compounds, however, depends on factors such as the availability of precursors, temperature, time, and water activity.

Production of process flavor concentrates is accomplished by selecting reaction mixtures and conditions so that those reactions occurring in normal food processing are duplicated. Selected ingredients (Table 10.5), usually including a reducing sugar, amino acids, and compounds with sulfur atoms, are processed under elevated temperatures to produce a distinctive profile of flavor compounds [35]. Thiamine is a popular ingredient because it provides both nitrogen and sulfur atoms already in ring structures (see Chapter 7).

Because of the large number of process flavor compounds produced during normal food processing or process simulation, it is unrealistic to cover the chemistry of their formation in depth. Rather, examples are given to illustrate some of the more important flavor volatiles formed and the mechanisms of their formation. Alkyl pyrazines were among the first compounds to be recognized as important contributors to the flavors of all roasted, toasted or similarly thermally processed foods. The most direct route to their formation results from the interaction of α-dicarbonyl compounds (intermediate products in the Maillard reaction) with amino acids through the Strecker degradation reaction (Figure 10.33). Transfer of the amino group to the dicarbonyl provides a means for integrating amino acid nitrogen into small compounds destined for any of the condensation reaction mechanisms envisioned in these reactions. Methionine has been selected as the amino acid involved

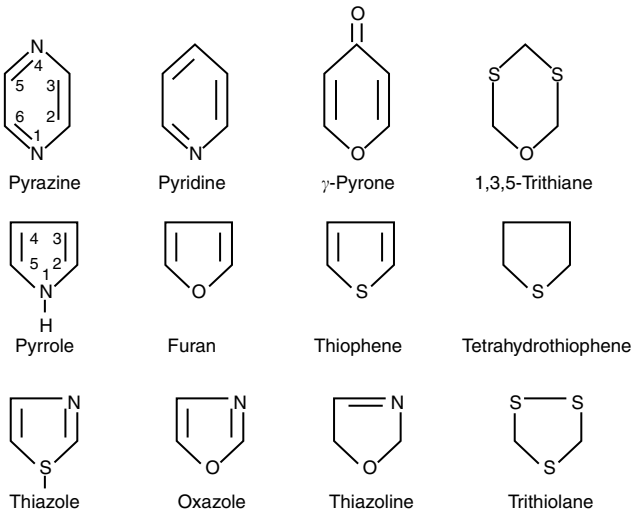


FIGURE 10.32 Some heterocyclic molecular skeletons found commonly in flavor compounds associated with thermally induced or browning flavors.

TABLE 10.5
Some Common Ingredients Used in Process Flavor Reaction Systems for the Development of Meat-like Flavors

Hydrolyzed Vegetable Protein	Thiamine
Yeast autolysate	Cysteine
Beef extract	Glutathione
Specific animal fats	Glucose
Chicken egg solids	Arabinose
Glycerol	5′-Ribonucleotides
Monosodium glutamate	Methionine

in the Strecker degradation reaction because it contains a sulfur atom and it leads to formation of methional, which is an important character-impact compound in boiling potatoes and cheese–cracker flavors. Methional also readily decomposes further to yield methanethiol (methyl mercaptan) that oxidizes to dimethyl disulfide, thus providing a source of reactive, low molecular weight sulfur compounds that contribute to the overall system of flavor development.

Hydrogen sulfide and ammonia are very reactive ingredients in mixtures intended for the development of process flavors, and they are often included in model systems and assist in promoting certain reaction mechanisms. Thermal degradation of cysteine (Figure 10.34) yields both ammonia and hydrogen sulfide as well as acetaldehyde. Subsequent reaction of acetaldehyde with a mercapto derivative of acetoin (from the Maillard reaction) gives rise to thiazoline that contributes to the flavor of boiled beef [57].

Some heterocyclic flavor compounds are quite reactive and tend to degrade or interact further with components of foods or reaction mixtures. An interesting example of flavor stability and carry through in foods is provided by the compounds shown in Equation 10.2, both of which provide

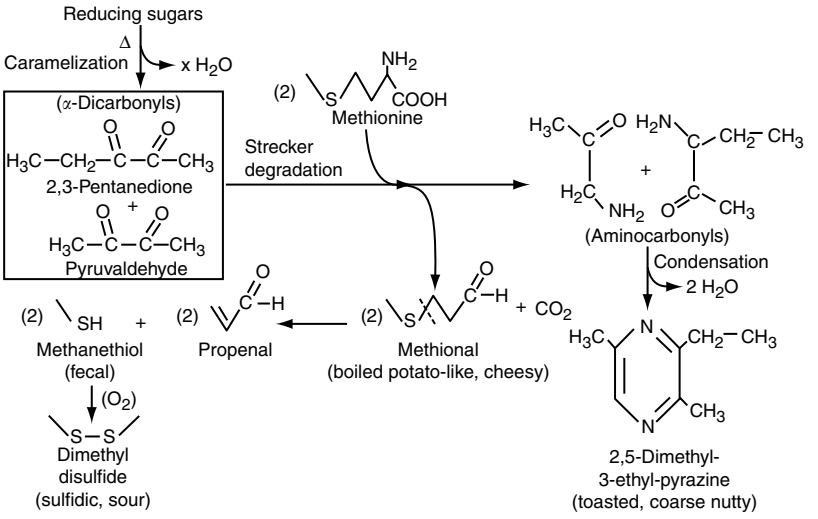


FIGURE 10.33 Formation of an alkyl pyrazine and small sulfur compounds through reactions occurring in the development of process flavors.

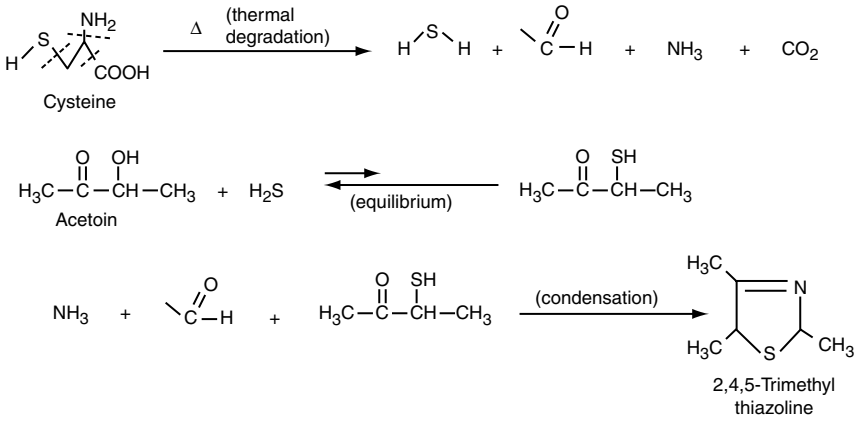
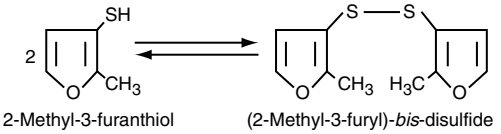


FIGURE 10.34 Formation of a thiazoline found in cooked beef through the reaction of fragments from cysteine and sugar-amino browning.

distinct, but different meat-like aromas [20]. A roasting meat aroma is exhibited by



(10.2)

2-methyl-3-furanthiol (reduced form), but upon oxidation to the disulfide form, the flavor becomes more characteristic of fully cooked meat that has been held for some time. Chemical reactions, such as the one just mentioned, are responsible for the subtle changes in meat flavor that occur because of the degree of cooking and the time interval after cooking.

During processing of complex systems, sulfur as such, or as thiols or polysulfides, can be incorporated in various compounds, resulting in the generation of new flavors. However, even

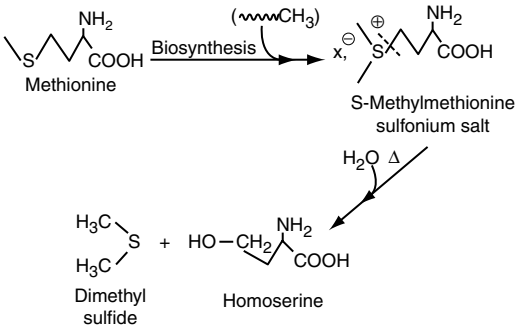


FIGURE 10.35 Formation of dimethyl sulfide from thermal degradation of *S*-methylmethionine sulfonium salts.

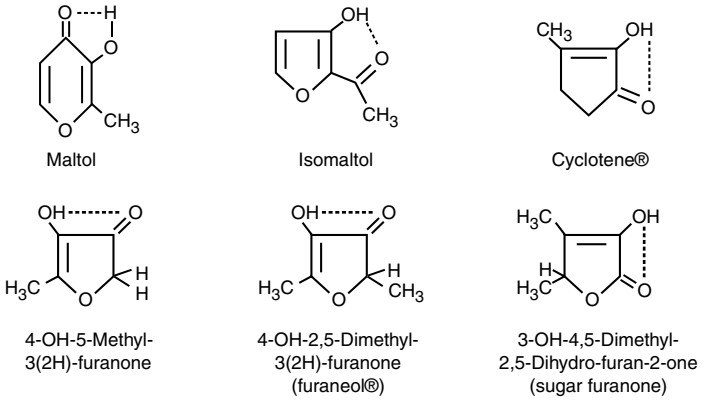


FIGURE 10.36 Structures of some important caramel-like flavor compounds derived from reactions occurring during processing.

though dimethyl sulfide is often found in processed foods, it does not react readily. In plant foods, dimethyl sulfide originates from biologically synthesized molecules, especially *S*-methylmethionine sulfonium salts (Figure 10.35). *S*-Methylmethionine salts are quite labile to heat, and dimethyl sulfide is readily released during cooking. In the case of plant-derived foods, dimethyl sulfide provides especially characterizing top notes to the flavors of freshly boiled and canned sweet corn as well as to tomato juice and other cooked tomato products.

Some of the most pleasant aromas derived from process reactions are provided by the compounds shown in Figure 10.36. These compounds exhibit caramel-like aromas and have been found in many processed foods. Cyclotene is used widely as a synthesized maple syrup flavor substance, and maltol is used widely as a flavor enhancer for sweet foods and beverages (Section 10.2.6). 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol) is sometimes known as the "pineapple compound" because it was first isolated from processed pineapple, where it contributes strongly to its characteristic flavor. Furaneol is also produced in biological systems, and it contributes a very ripe strawberry note that can be detected over freshly picked fruit. 3-OH-4,5-Dimethyl-2,5-dihydrofuran-2-one is often referred as sugar furanone because its highly characterizing aroma can easily be detected in the gases above bulk refined sugars (sucrose). Additionally, furanones are found in cooked meats, such as boiled beef, where they apparently enhance meatiness.

The planar enol-cyclic-ketone structure for the compounds shown in Figure 10.36 is typically derived from the sugar precursors, and this structural component appears responsible for their caramel-like aroma quality [61]. As shown in Equation 10.3 for maltol, the planar enolone form is

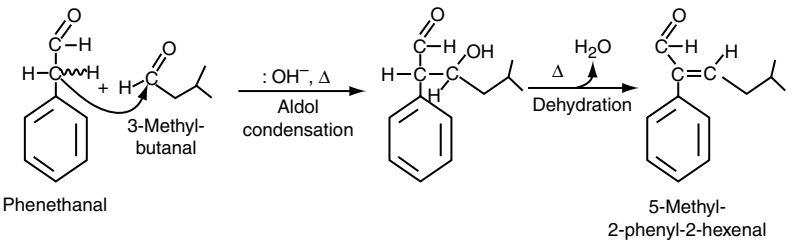
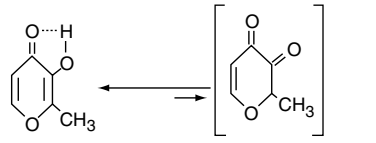


FIGURE 10.37 Formation of an important cocoa aroma volatile through an aldol condensation of two Strecker reaction-derived aldehydes.

largely favored over the cyclic diketone form because the enolone form allows strong intramolecular H-bonding to occur.



(10.3)

The flavor of chocolate and cocoa has received much attention because of the high demand for these flavors. After harvesting, cocoa beans are often fermented under somewhat poorly controlled conditions. The beans are then roasted, sometimes with an intervening alkali treatment that darkens the color and yields a less harsh flavor. The fermentation hydrolyzes sucrose to reducing sugars, frees amino acids, and oxidizes some polyphenols. During roasting, many pyrazines and other heterocyclics are formed, but the unique flavor of cocoa is derived from an interaction between aldehydes from the Strecker degradation reaction. The reaction shown in Figure 10.37 between phenylacetaldehyde (from phenylalanine) and 3-methylbutanal (from leucine) constitutes an important flavor-forming reaction in cocoa. The product of this aldol condensation, 5-methyl-2-phenyl-2-hexenal, exhibits a character-impact persistent chocolate aroma. This example also serves to show that reactions in the development of process flavors do not always yield heterocyclic aroma compounds.

10.7.2 Volatiles Derived from Oxidative Cleavages of Carotenoids

Oxidations focusing on triacylglycerols and fatty acids were discussed in previous sections, but some extremely important flavor compounds that are oxidatively derived from carotenoid precursors have not been covered and deserve mention here. Some of these reactions require singlet oxygen through chlorophyll sensitization; others are photooxidation processes. A large number of flavor compounds, derived from oxidizing carotenoids (or isoprenoids), have been identified in curing tobacco [18], and many of these are considered important for characterizing tobacco flavors. However, relatively few compounds in this category (three representative compounds are shown in Figure 10.38) are currently considered highly important as food flavors. Each of these compounds exhibits unique sweet, floral, and fruit-like characteristics that vary greatly with concentration. They also blend nicely with aromas of foods to produce subtle effects that may be highly desirable or very undesirable. β-Damascenone exerts very positive effects on fresh apple aromas. It also enhances the flavor of wines, but in beer this compound at only a few parts per billion results in a stale, raisin-like note. β-Ionone also exhibits a pleasant violet, floral aroma compatible with fruit-type flavors, but it is also the principal off-flavor compound present in oxidized, freeze-dried carrots. Furthermore, these compounds have been found in black tea, where they make positive contributions to the flavor. Theaspirane and related derivatives

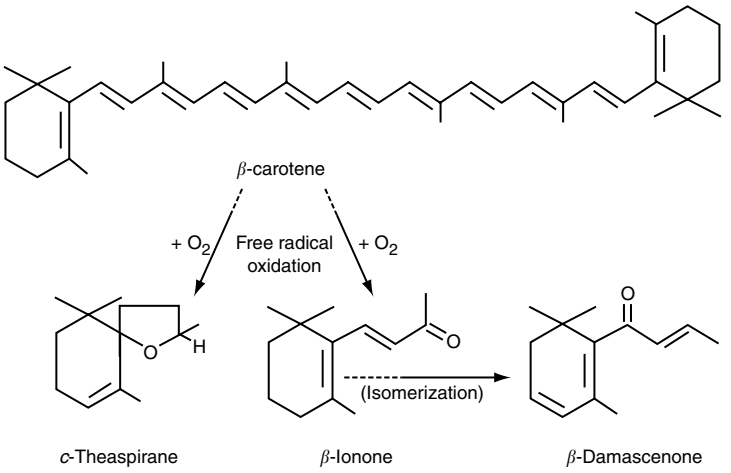


FIGURE 10.38 Formation of some important tea flavor compounds through oxidative cleavages of carotenoids.

contribute importantly to the sweet, fruity, and earthy notes of tea aroma. Although usually present in low concentrations, these compounds and related ones appear to be widely distributed, and it is likely that they contribute to the full, well-blended flavors of many foods.

10.8 FUTURE DIRECTIONS OF FLAVOR CHEMISTRY AND TECHNOLOGY

Much has been accomplished over the past 45 years since the golden era of modern flavor chemistry and technology [78] was initiated with the development and ready availability of powerful gas chromatography and fast-scan mass spectrometry instruments. Overall, much of this period was devoted to the systematic discovery of aroma-active substances, and aside from ferreting out details, focused efforts have largely accomplished the objectives of the task. Similarly, the knowledge base surrounding the mechanistic aspects involved in the formation and degradation of flavor compounds has become extensive, but many details of these processes still remain incomplete and available for further fruitful study. Certainly, the continued advancement of genetic engineering for specific flavor effects in various single-cell and higher organisms serves to provide motivations for additional efforts to more thoroughly understand the intricacies of mechanisms involved in the biosynthesis of flavor-related substances.

Nevertheless, it is likely that the focus of future flavor chemistry research will need to shift considerably to meet the most pressing demands facing the food industry. Substantial expansions of nutraceutical or functional foods will bring new challenges to mask or otherwise neutralize undesirable flavors that are inherent in ingredients employed to provide the functional properties. However, the most challenging problems reside in the successful reformulation of foods for health reasons to avoid excessive amounts of more traditional ingredients, particularly salt, fats, and refined carbohydrates. Initial attempts to develop what have been called "nutritionally appropriate" foods have met with many failures and frustrations, but public pressures are building to make the food industry overcome hurdles that stymied earlier attempts to provide these foods.

Recent flavor chemistry studies on rates of release of individual flavor chemicals from various matrixes were carried out in an attempt to understand and overcome problems encountered in providing high-quality flavors in reformulated foods whose compositions have been radically changed to meet a nutrition goal (e.g., fat elimination, etc.). Still, to date, breakthrough findings have not

emerged from flavor release studies, which suggest that other approaches also need to be vigorously explored. One of the most overlooked areas in this regard is that of flavor enhancers and modifiers (Section 10.2.6) whose roles in food palatability are not well recognized nor well understood, and the area is ripe for exploration. Such efforts, however, will require changes in traditional food and flavor chemistry thinking, and necessarily also will require alterations in sensory analysis techniques to specifically detect and assess the subtle food palatability characteristics provided by flavor modifiers. The challenges are great, but the potential benefits to health and well-being of the population also are great.

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11 Food Additives

Robert C. Lindsay

CONTENTS

11.1 INTRODUCTION

Many substances are incorporated into foods for functional purposes, and in many cases these ingredients can also be found occurring naturally in some food. However, when they are used in processed foods, these chemicals have become known as "food additives." From a regulatory standpoint, each of the food additives must provide some useful and acceptable function or attribute to justify its usage. Generally, improved keeping quality, enhanced nutritional value, functional property provision and improvement, processing facilitation, and enhanced consumer acceptance are considered acceptable functions for food additives. The use of food additives to conceal damage or spoilage to foods or to deceive consumers is expressly forbidden by regulations governing the use of these substances in foods. Additionally, food additive usages are discouraged where similar effects can be obtained by economical, good manufacturing practices.

The breadth of food constituents that can be considered to be of suitable importance for specific attention in food chemistry textbooks has expanded in recent years, and some of these constituents can be handled in a manner similar to traditional food additives. However, increased understandings of health benefits provided by individual or groups of food constituents have prompted commercial introductions of a broad range of new food ingredients. Many of these ingredients have been developed to meet formulation challenges presented by specific needs, such as fat calorie reductions, while others have emerged as ingredients intended to provide specific health benefits, for example, plant sterols for blood cholesterol reduction. While many of these ingredients and substances clearly fall into the food additives category, a movement is underway to disassociate them from the unfavorable image of food additives.

Some blurring of traditional terminology already has occurred. Notable is the recent widespread acceptance of the terms, functional foods and functional food ingredients, to designate foods and ingredients containing health-related constituents in greater abundance than usual counterparts. As a result of this usage, the utility of the terms, functional purposes and functional food ingredients, as they have been applied traditionally to food additive performances has been diminished, and alternative terminology needs to be explored.

Nevertheless, natural counterparts exist for many food additives, and increasingly new ingredients themselves are derived commercially from natural sources. This chapter focuses on the role of both natural and synthetic substances that are added to foods and provides an integrating view of their functionalities. Further discussions about the chemistry of specific food components often can be found in the appropriate primary constituent chapters of this book. For example, natural substances sometimes used as food additives are discussed in Chapter 4 (Section 4.7.4, Antioxidants), Chapter 7 (Vitamins), Chapter 8 (Minerals), Chapter 9 (Colorants), Chapter 10 (Flavors), and Chapter 12 (Nutraceuticals).

11.2 ACIDS

11.2.1 General Attributes

Both organic and inorganic acids occur extensively in natural systems where they function in a variety of roles ranging from intermediary metabolites to components of buffer systems. Acids are added for numerous purposes in foods and food processing where they provide the benefits of many of their natural actions. One of the most important functions of acids in foods is participation in buffering systems, and this aspect is discussed in the following section. The use of acids and acid salts in chemical leavening systems, the role of specific acidic microbial inhibitors (e.g., sorbic acid, benzoic acid) in food preservation, and the function of acids as chelating agents are also discussed in subsequent sections of this chapter. Acids are important in the setting of pectin gels (Chapter 3), they serve as defoaming agents and emulsifiers, and they induce coagulation of milk proteins (Chapters 5 and 15) in the production of cheese and cultured dairy products such as sour cream. In natural culturing processes, lactic acid (CH_3—CHOH—COOH) produced by streptococci and lactobacilli causes coagulation by lowering the pH to near the isoelectric point of casein. Cheeses can be produced by adding rennet and acidulants such as citric acid and hydrochloric acids to cold milk (4–8°C). Subsequent warming of the milk (to 35°C) produces a uniform gel structure. Addition of acid to warm milk results in a protein precipitate rather than a gel.

δ-Gluconolactone that is derived from glucose via a process involving fermentation also can be used for slow acid production in cultured dairy products and chemical leavening systems because it slowly hydrolyzes in aqueous systems to form gluconic acid (Figure 11.1). Dehydration of lactic acid yields lactide, a cylic dilactone (Figure 11.2), that also can be used as a slow-release acid in aqueous systems. The dehydration reaction occurs under conditions of low water activity and elevated temperature. Introduction of lactide into foods with high water activity causes a reversal of the process with the production of two moles of lactic acid.

Acids such as citric are added to some moderately acidic fruits and vegetables to lower the pH to a value below 4.5. In canned foods, this permits sterilization to be achieved under less severe thermal

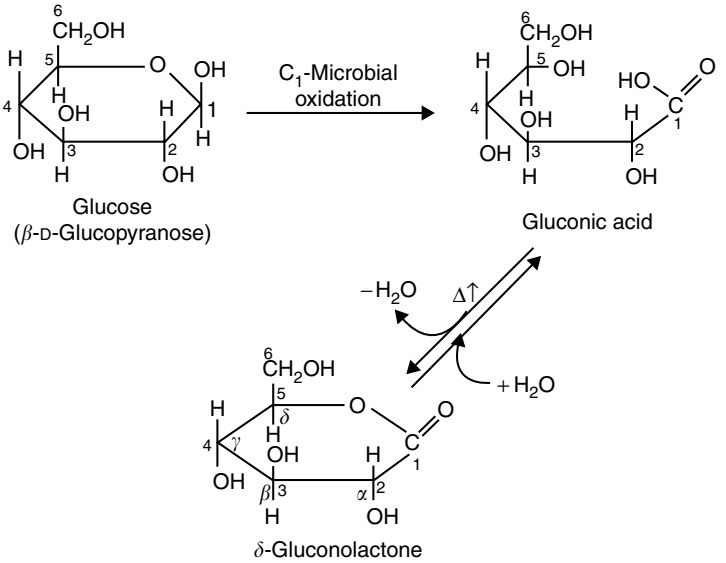


FIGURE 11.1 Reactions for the formation of gluconic acid via fermentative oxidation of glucose followed by thermal dehydration to form δ-gluconolactone; the latter reaction is reversed on contact of δ-gluconolactone, with water providing a means for slow-release acidification of foods.

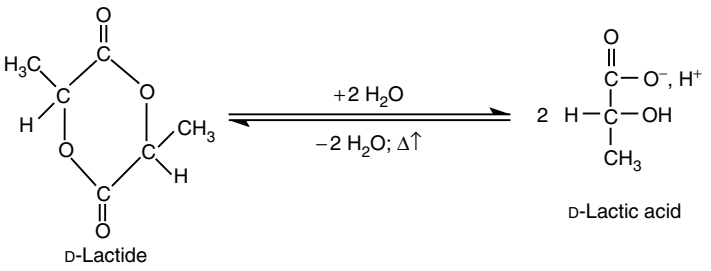


FIGURE 11.2 Equilibrium reaction showing formation of lactic acid from the hydrolysis of lactide.

conditions than is necessary for less acidic products and has the added advantage of precluding the growth of hazardous microorganisms (i.e., *Clostridium botulinum*).

Acids, such as potassium acid tartrate (HOOC—CHOH—CHOH—COOK), are employed in the manufacture of fondant and fudge to induce limited hydrolysis (inversion) of sucrose (Chapter 3). Inversion of sucrose yields fructose and glucose, which improve texture through inhibition of excessive growth of sucrose crystals. Monosaccharides inhibit sucrose crystallization by contributing to the complexity of the syrup and by lowering its water activity and corresponding equilibrium relative humidity.

One of the most important contributions of acids to foods is their ability to produce a sour or tart taste ([22]; Chapter 10). Acids also have the ability to modify and intensify the taste perception of other flavoring agents. The hydrogen ion or hydronium ion (H_3O^+) is involved in the generation of the sour taste response. Furthermore, short-chain free fatty acids (C_2—C_{12}) contribute significantly to the aroma of foods. For example, acetic acid dominates the aroma and taste (flavor) of vinegar. Butyric acid, at relatively high concentrations, contributes strongly to the characteristic flavor of hydrolytic rancidity in dairy products, but at lower concentrations contributes to the typical flavor of foods such as cheese and butter.

The ability to influence the pH of foods obviously also is an important consideration in the selection of an acid for a specific application, and this is governed largely by the extent of dissociation experienced by the acid functional group in aqueous systems. Dissociation constants for some acids used in foods are shown in Table 11.1 [59]. Some of the more commonly used organic acids for food applications are acetic (CH_3COOH), lactic (CH_3—CHOH—COOH), citric (HOOC—CH_2—COH(COOH)—CH_2—COOH), malic (HOOC—CHOH—CH_2—COOH), fumaric (HOOC—CH═CH—COOH), succinic (HOOC—CH_2—CH_2—COOH), and tartaric (HOOC—CHOH—CHOH—COOH). Phosphoric acid (H_3PO_4) is the most widely used inorganic acid food acidulant, and it is employed extensively in flavored carbonated beverages, particularly in colas and root beer.

The strong mineral acids (e.g., HCl, H_2SO_4) are often too highly dissociable for general food acidulation, and their direct introduction may lead to problems with quality attributes of foods. However, the highly dissociable mineral acids are useful and economic in the manufacture of some intermediate ingredients, such as protein hydrolysates. Sodium acid sulfate (e.g., sodium bisulfate; $NaHSO_4$) or half-neutralized sulfuric acid provides another ingredient option, and has recently received U.S. GRAS (generally recognized as safe) approval. Sodium acid sulfate is manufactured in a granular powder form, and its pK_a allows it to provide acidulation properties similar to phosphoric acid.

11.2.2 Chemical Leavening Systems

Chemical leavening systems are composed of compounds that react to release gas in a dough or batter under appropriate conditions of moisture and temperature. During baking, this gas release, along with expansion of entrapped air and moisture vapor, imparts a characteristic porous, cellular structure to finished goods. Chemical leavening systems are found in self-rising flours, prepared baking mixes, household and commercial baking powders, and refrigerated dough products [25].

TABLE 11.1
Dissociation Constants at 25°C for Some Acids Used in Foods

Acid	Step	pK_a	Acid	Step	pK_a
Organic acids					
Acetic		4.75	Propionic		4.87
Adipic	1	4.43	Succinic	1	4.16
	2	5.41		2	5.61
Benzoic		4.19	Tartaric	1	3.22
n-Butyric		4.81		2	4.82
Citric	1	3.14	**Inorganic acids**		
	2	4.77	Carbonic	1	6.37
	3	6.39		2	10.25
Formic		3.75	*o*-Phosphoric	1	2.12
Fumaric	1	3.03		2	7.21
	2	4.44		3	12.67
Hexanoic		4.88	Pyrophosphoric	1	0.85
Lactic		3.08		2	1.49
Malic	1	3.40		3	5.77
	2	5.10		4	8.22
			Sulfuric	2	1.92

Source: From Weast, R. C. (ed.) (1988). *Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL, pp. D161–D163.

Carbon dioxide is the only gas generated from currently used chemical leavening systems, and it is derived from a carbonate or bicarbonate salt. The most common leavening salt is sodium bicarbonate ($NaHCO_3$), although ammonium carbonate [$(NH_4)_2CO_3$] and bicarbonate (NH_4HCO_3) are sometimes used in cookies. Both of the ammonium salts decompose at baking temperatures, and thus do not require, as does sodium bicarbonate, an added leavening acid for functionality. Potassium bicarbonate ($KHCO_3$) has been employed as a component of leavening systems in reduced-sodium diets, but its application is somewhat limited because of its hygroscopic nature and slight bitter flavor.

Sodium bicarbonate is quite soluble in water (619 g/l00 mL), and ionizes completely (Equations 11.1, 11.2, and 11.3).

$$NaHCO_3 \rightleftharpoons Na^+ + HCO_3^- \tag{11.1}$$

$$HCO_3^- + H_2O \rightleftharpoons H_2CO_3 + OH^- \tag{11.2}$$

$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+ \tag{11.3}$$

These reactions, of course, apply only to simple water solutions. In dough systems, the ionic distribution becomes much more complex since proteins and other naturally occurring ionic species are available to participate in the reactions. In the presence of hydrogen ions provided mainly by leavening acids, and to some extent by the dough, sodium bicarbonate reacts to release carbon dioxide (Equation 11.4; showing a carboxyl acid).

$$R{-}COO^-, H^+ + NaHCO_3 \rightleftharpoons R{-}COO^-, Na^+ + H_2O + CO_2 \uparrow \tag{11.4}$$

The proper balance of acid and sodium bicarbonate are essential because excess sodium bicarbonate imparts a soapy taste to bakery products; an excess of acid leads to tartness and sometimes bitterness. The neutralizing power of leavening acids is not uniform and the relative activity of an acid is given by

its neutralizing value, which is determined by calculating the parts by weight of sodium bicarbonate that will neutralize l00 parts by weight of the leavening acid [53]. However, in the presence of natural flour ingredients, the amount of leavening acid required to give neutrality or any other desired pH in a baked product may be quite different from the theoretical amount determined for a simple system. Still, neutralizing values are useful in determining initial formulations for leavening systems. Residual salts from a properly balanced leavening process help stabilize the pH of finished products.

Leavening acids are often not easily recognized as acids in the usual sense, yet they must provide hydrogen ions to release carbon dioxide. The phosphates and potassium acid tartrate are salts of partially neutralized acids; sodium aluminum sulfate reacts with water to yield sulfuric acid (Equation 11.5). As mentioned earlier, δ-gluconolactone is an intramolecular ester (or lactone) that hydrolyzes slowly in aqueous systems to yield gluconic acid.

$$Na_2SO_4 \cdot Al_2(SO_4)_3 + 6H_2O \rightleftharpoons Na_2SO_4 + 2Al(OH)_3 + 3H_2SO_4 \tag{11.5}$$

Leavening acids generally exhibit limited water solubility at room temperature, but some are less soluble than others. This difference in solubility or availability accounts for the initial rate of carbon dioxide release at room temperature and is the basis for classifying leavening acids according to speed. For example, if the compound is moderately soluble, carbon dioxide is rapidly evolved and the acid is referred to as fast-acting. Conversely, if the acid dissolves slowly, it is a slow-acting leavening acid. Leavening acids usually release a portion of the carbon dioxide before baking and the remainder under elevated temperatures of the baking process.

General patterns of carbon dioxide release at 27°C for fast-acting monocalcium phosphate monohydrate [$Ca(H_2PO_4)_2 \cdot H_2O$] and slow-acting 1-3-8 sodium aluminum phosphate [$NaH_{14}Al_3(PO_4)_8 \cdot 4H_2O$] are shown in Figure 11.3 [53]. Over 60% of the carbon dioxide is released very quickly from the more soluble monocalcium phosphate monohydrate; only 20% of the potential carbon dioxide is released from the slow-acting 1-3-8 sodium aluminum phosphate during a 10 min reaction period. Because of a hydrated alumina coating the latter leavening acid reacts to only a small extent until activated by heat. Also shown in Figure 11.3 is the low temperature release pattern of carbon dioxide from coated anhydrous monocalcium phosphate [$Ca(H_2PO_4)_2$]. The crystals of this leavening acid were coated with compounds of slightly soluble alkali metal phosphates. The gradual release of carbon dioxide over the 10 min reaction period corresponds to the time required for water to penetrate the coating. This behavior is very desirable in some products that encounter a delay prior to baking.

The release of the remainder of the carbon dioxide from leavening systems during baking provides the final modifying action on texture. In most leavening systems, the rate at which carbon dioxide is released greatly accelerates as the temperature is elevated. The effect of elevated temperatures on the release rate of carbon dioxide from slow-acting sodium acid pyrophosphate ($Na_2H_2P_2O_7$) is presented in Figure 11.4. Even a slight increase in temperature (from 27°C to 30°C) noticeably accelerates gas production. Temperatures near 60°C cause a complete release of carbon dioxide within 1 min. Some leavening acids are less sensitive to high temperatures and do not exhibit vigorous activity until temperatures near the maximum baking temperature are obtained. Dicalcium phosphate ($CaHPO_4$) is unreactive at room temperature because it forms a slightly alkaline solution at this temperature. However, upon heating above approximately 60°C, hydrogen ions are released, thereby activating the leavening process. This slow action confines its use to products requiring long baking times, such as some types of cakes. Formulations of leavening acids employing one or more acidic components are common, and systems are often tailored for specific dough or batter applications.

Leavening acids currently employed include potassium acid tartrate, sodium aluminum sulfate, δ-gluconolactone, and ortho- and pyrophosphates. The phosphates include calcium phosphate, sodium aluminum phosphate, and sodium acid pyrophosphate. Some general properties of commonly

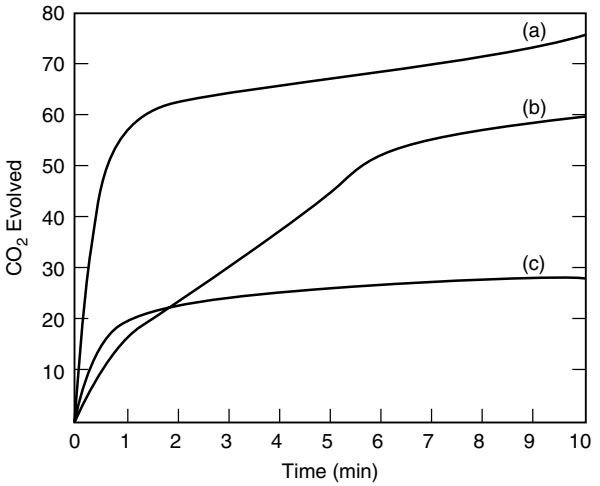


FIGURE 11.3 Carbon dioxide production at 27°C from the reaction of $NaHCO_3$ with (a) monocalcium phosphate · H_2O, (b) coated anhydrous monocalcium phosphate, and (c) 1-3-8 sodium aluminum phosphate. (Data from Stahl, J. E. and R. H. Ellinger (1971). In *Symposium: Phosphates in Food Processing* (J. M. Deman and P. Melnychyn, eds.), AVI Publishing Co., Westport, CO, pp. 194–212.)

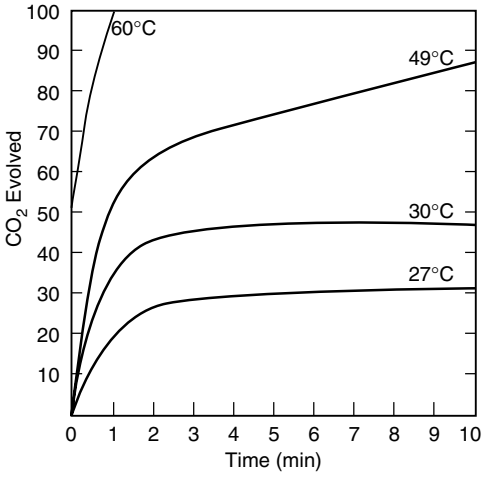


FIGURE 11.4 Effect of temperature on the rate of carbon dioxide evolution from the reaction of $NaHCO_3$ and slow-speed acid pyrophosphate. (Reprinted from Stahl, J. E. and R. H. Ellinger (1971). In *Symposium: Phosphates in Food Processing* (J. M. Deman and P. Melnychyn, eds.), AVI Publishing Co., Westport, CO, p. 201.)

used leavening acids are given in Table 11.2. It must be remembered that these are only examples, and that an extensive technology has developed for modification and control of the phosphate leavening acids [53].

Baking powders account for a large part of the chemical leaveners used both in the home and in bakeries. These preparations include sodium bicarbonate, suitable leavening acids, and starch and other extenders. Federal standards for baking powder require that the formula must yield at least 12% by weight of available carbon dioxide, and most contain 26–30% by weight of sodium bicarbonate. Traditional baking powders of the potassium acid tartrate type have been largely replaced by double-acting preparations. In addition to $NaHCO_3$ and starch, these baking powders usually

TABLE 11.2
Some Properties of Common Leavening Acids

Acid	Formula	Neutralizing Value[a]	Relative Reaction Rate at Room Temperature[b]
Sodium aluminum sulfate	$Na_2SO_4 \cdot Al_2(SO_4)_3$	100	Slow
Dicalcium phosphate dihydrate	$CaHPO_4 \cdot 2H_2O$	33	None
Monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2 \cdot H_2O$	80	Fast
l-3-8 Sodium aluminum phosphate	$NaH_{14}Al_3(PO_4)_8 \cdot 4H_2O$	100	Slow
Sodium acid pyrophosphate (slow-type)	$Na_2H_2P_2O_7$	72	Slow
Potassium acid tartrate	$KHC_4H_4O_6$	50	Medium
δ-Gluconolactone	$C_6H_{10}O_6$	55	Slow

[a] In simple model systems; parts by weight of $NaHCO_3$ that will neutralize l00 parts by weight of the leavening acid.
[b] Rate of CO_2 evolution in the presence of $NaHCO_3$.

Source: From Stahl, J. E. and R. H. Ellinger (1971). In *Symposium: Phosphates in Food Processing* (J. M. Deman and P. Melnychyn, eds.), AVI Publishing Co., Westport, CO, pp. 194–212.

contain monocalcium phosphate monohydrate [$Ca(H_2PO_4)_2 \cdot H_2O$], which provides rapid action during the mixing stage, and sodium aluminum sulfate [$Na_2SO_4 \cdot Al_2(SO_4)_3$], which does not react appreciably until the temperature increases during baking.

The increase in convenience foods has stimulated sales of prepared baking mixes and refrigerated dough products. In white and yellow cake mixes, the most widely used blend of leavening acids contains anhydrous monocalcium phosphate [$Ca(H_2PO_4)_2$] and sodium aluminum phosphate [$NaH_{14}Al_3(PO_4)_8 \cdot 4H_2O$]; chocolate cake mixes usually contain anhydrous monocalcium phosphate and sodium acid pyrophosphate ($Na_2H_2P_2O_7$) [53]. Typical blends of acids contain 10–20% fast-acting anhydrous monophosphate and 80–90% of the slower acting sodium aluminum phosphate or sodium acid pyrophosphate compounds. The leavening acids in prepared biscuit mixes usually consist of 30–50% anhydrous monocalcium phosphate and 50–70% sodium aluminum phosphate or sodium acid pyrophosphate. The earliest self-rising flours and corn meal mixes contained monocalcium phosphate monohydrate [$Ca(H_2PO_4)_2 \cdot H_2O$], but coated anhydrous monocalcium phosphate and sodium aluminum phosphate are in common use [53].

Refrigerated doughs for biscuit and roll products require limited initial carbon dioxide release during preparation and packaging, and considerable gas release during baking. Formulations for biscuits usually contain from 1.0% to 1.5% sodium bicarbonate and 1.4% to 2.0% slow-acting leavening acids, such as coated monocalcium phosphate and sodium acid pyrophosphate, based on total dough weight. The pyrophosphates are useful in dough because they can be manufactured with a wide range of reactivities. For example, pyrophosphatase in flour is capable of hydrolyzing sodium acid pyrophosphate to orthophosphate (Figure 11.5), and the reaction of sodium bicarbonate and pyrophosphate yields some trisodium monohydrogen pyrophosphate, which also can be hydrolyzed to orthophosphates. This enzymic action leads to gas production that assists in sealing packages of refrigerated dough, but it can also lead to formation of large crystals of orthophosphates that may be mistaken for broken glass by the consumer.

11.3 BASES

Basic or alkaline substances are used in a variety of applications in foods and food processing. Although the majority of applications involve buffering and pH adjustments, other functions include carbon dioxide evolution, enhancement of color and flavor, solubilization of proteins, and chemical

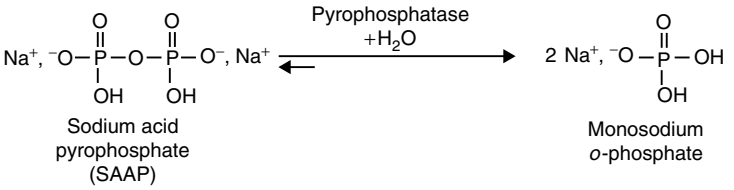


FIGURE 11.5 Enzymic hydrolysis of sodium acid pyrophosphate.

peeling. The role of carbonate and bicarbonate salts in carbon dioxide production during baking has been discussed previously.

Alkali treatments are imposed on several food products for the purpose of color and flavor improvement. Ripe olives are treated with solutions of sodium hydroxide (0.25–2.0%) to aid in the removal of the bitter principal and to develop a darker color. Pretzels are dipped in a solution of 1.25% sodium hydroxide at 87–88°C (186–190°F) before baking to alter proteins and starch so that the surface becomes smooth and develops a deep-brown color during baking. It is believed that the NaOH treatment used to prepare hominy and tortilla dough destroys disulfide bonds that are base labile and improves the flavor. Soy proteins are solubilized through alkali processing and concern has been expressed about alkaline-induced racemization of amino acids (Chapter 5) and losses of other nutrients. Small amounts of sodium bicarbonate are used in the manufacture of peanut brittle candy to enhance caramelization and browning, and to provide, through release of carbon dioxide, a somewhat porous structure. Bases, usually potassium carbonate, are also used in cocoa processing for the production of dark (Dutched) chocolate [41]. The elevated pH enhances sugar–amino browning reactions and polymerization of flavonoids (Chapter 9) resulting in a smoother, less acid and less bitter chocolate flavor, a darker color, and a slightly improved solubility.

Food systems sometimes require adjustment to higher pH values to achieve more stable or more desirable characteristics. For example, alkaline salts, such as disodium phosphate, trisodium phosphate, and trisodium citrate are used in the preparation of processed cheese (1.5–3%) to increase the pH (from 5.7 to 6.3), and to effect protein (casein) dispersion. This salt–protein interaction improves the emulsifying and water-binding capabilities of the cheese proteins [48] because the salts bind the calcium components of the casein micelles forming chelates.

Instant milk-gel puddings are prepared by combining dry mixes containing pregelatinized starch with cold milk and allowing them to stand for a short time at refrigerator temperatures. Alkaline salts, such as tetrasodium pyrophosphate ($Na_4P_2O_7$) and disodium phosphate (Na_2HPO_4), in the presence of calcium ions in milk cause milk proteins to gel in combination with the pregelatinized starch. The optimum pH for acceptable puddings falls between 7.5 and 8.0. Although some of the necessary alkalinity is contributed by alkaline phosphate salts, other alkalizing agents are often added [13].

The addition of phosphates and citrates changes the salt balance in fluid milk products by forming complexes with calcium and magnesium ions from casein. The mechanism is incompletely understood, but depending on the type and concentration of salt added, the milk protein system can undergo stabilization, gelation, or destabilization.

Alkaline agents are used to neutralize excess acid in the production of such foods as cultured butter. Before churning, the cream is fermented by lactic acid bacteria so that it contains about 0.75% titratable acidity expressed as lactic acid. Alkalis are then added to achieve a titratable acidity of approximately 0.25%. The reduction in acidity improves churning efficiency and retards the development of oxidative off-flavors. Several materials, including sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), magnesium carbonate ($MgCO_3$), magnesium oxide (MgO), calcium hydroxide ($Ca(OH)_2$), and sodium hydroxide (NaOH) are utilized alone or in combination as neutralizers for foods. Solubility, foaming as a result of carbon dioxide release, and strength of the base influence the selection of the alkaline agent. The use of alkaline agents or bases in excessive amounts leads to soapy or neutralizer flavors, especially when substantial quantities of fatty acids are present.

Strong bases are employed for peeling various fruits and vegetables. Exposure of the product to hot solutions 60–82°C (140–180°F) of sodium hydroxide (about 3%), with subsequent mild abrasion, effects peel removal with substantial reductions in plant wastewater as compared with other conventional peeling techniques. Differential solubilization of cell and tissue constituents (pectic substances in the middle lamella are particularly soluble) provides the basis for caustic peeling processes.

11.4 BUFFER SYSTEMS AND SALTS

11.4.1 Buffers and pH Control in Foods

Since most foods are complex materials of biological origin, they contain many substances that can participate in pH control and buffering systems—included are proteins, organic acids, and weak inorganic acid phosphate salts. Lactic acid and phosphate salts, along with proteins, are important for pH control in animal tissue; polycarboxylic acids, phosphate salts, and proteins are important in plant tissues. The buffering effects of amino acids and proteins and the influence of pH and salts on their functionalities are discussed in Chapter 5. In plants, buffering systems containing citric acid (lemons, tomatoes, rhubarb), malic acid (apples, tomatoes, lettuce), oxalic acid (rhubarb, lettuce), and tartaric acid (grapes, pineapple) are common, and they usually function in conjunction with phosphate salts in maintaining pH control. Milk acts as a complex buffer because of its content of carbon dioxide, proteins, phosphate, citrate, and several other minor constituents.

In situations where the pH must be altered, it is usually desirable to stabilize the pH at the desired level through a buffer system. This is accomplished naturally when lactic acid is produced in cheese and pickle fermentations. Also, in some instances where substantial amounts of acids are used in foods and beverages, it is desirable to reduce the sharpness of acid tastes, and obtain smoother product flavors without inducing neutralization flavors. This usually can be accomplished by establishing a buffer system in which the salt of a weak organic acid is dominant. The common ion effect is the basis for obtaining pH control in these systems, and the system develops when the added salt contains an ion that is already present in an existing weak acid. The added salt immediately ionizes resulting in repressed ionization of the acid with reduced acidity and a more stable pH. The efficiency of a buffer depends on the concentration of the buffering substances. Since there is a pool of undissociated acid and dissociated salt, buffers resist changes in pH. For example, relatively large additions of a strong acid, such as hydrochloric acid, to an acetic acid–sodium acetate system causes hydrogen ions to react with the acetate-ion pool to increase the concentration of slightly ionized acetic acid, and the pH remains relatively stable. In a similar manner, an addition of sodium hydroxide causes hydroxyl ions to react with hydrogen ions to form undissociated water molecules.

Titration of buffered systems and resulting titration curves (i.e., pH vs. volume of base added) reveal their resistance to pH change. If a weak acid buffer is titrated with a base, there is a gradual but steady increase in the pH as the system approaches neutralization; that is, the change in pH per mL of added base is small. Weak acids are only slightly dissociated at the beginning of the titration. However, the addition of hydroxyl ions shifts the equilibrium to the dissociated species and eventually the buffering capacity is overcome.

In general, for an acid (HA) in equilibrium with ions (H^+) and (A^-), the equilibrium is expressed as in Equation 11.6:

$$HA \rightleftarrows H^+ + A^- \tag{11.6}$$

$$K_a^1 = \frac{[H^+][A^-]}{[HA]} \tag{11.7}$$

The constant K_a^1 is the apparent dissociation constant (Equation 11.7), and is characteristic of the particular acid. The apparent dissociation constant K_a^1 becomes equal to the hydrogen ion

concentration [H^+] when the anion concentration [A^-] becomes equal to the concentration of undissociated acid [HA]. This situation gives rise to an inflection point on a titration curve, and the pH corresponding to this point is referred to as the pK_a^1 of the acid. Therefore, for a weak acid, pH is equal to pK_a^1 when the concentration of the acid and conjugate base are equal (Equation 11.8):

$$pH = pK_a^1 = -\log_{10}[H^+] \tag{11.8}$$

A convenient method for calculating the approximate pH of a buffer mixture, given the pK_a^1 of the acid, is provided by Equation 11.11. This equation is arrived at first by solving Equation 11.7 for [H^+] to yield Equation 11.9.

$$[H^+] = K_a^1 \frac{[HA]}{[A^-]} = K_a^1 \frac{[\text{acid}]}{[\text{salt}]} \tag{11.9}$$

Since the salt in solution is almost completely dissociated, it is assumed equal to the concentration of the conjugate base [A^-]. The negative logarithms of the terms yield Equation 11.10. By substituting pH for $-\log [H^+]$ and pK_a^1 for $-\log K_a^1$, Equation 11.11 is obtained. The pH of a buffer system derived from any weak acid which dissociates to H^+ and A^- can be calculated by using Equation 11.11.

$$-\log[H^+] = -\log K_a^1 - \log \frac{[\text{acid}]}{[\text{salt}]} \tag{11.10}$$

$$pH = pK_a^1 + \log \frac{[\text{salt}]}{[\text{acid}]} = pK_a^1 + \log \frac{[A^-]}{[HA]} \tag{11.11}$$

In calculating the pH values of buffer solutions, it is important to recognize that the apparent dissociation constant K_a^1 differs from K_a, the true dissociation constant. However, for any buffer, the value of K_a^1 remains constant as the pH is varied, provided that the total ionic strength of the solution remains unchanged.

The sodium salts of gluconic, acetic, citric, and phosphoric acids are commonly used for pH control and tartness modification in the food industry. The citrates are usually preferred over phosphates for tartness modification since they yield smoother sour flavors. When low sodium products are required, potassium buffer salts may be substituted for sodium salts. In general, calcium salts are not used because of their limited solubilities and incompatibilities with other components in the system. The effective buffering ranges for combinations of common acids and salts are pH 2.1–4.7 for citric acid–sodium citrate, pH 3.6–5.6 for acetic acid–sodium acetate, and pH 2.0–3.0, 5.5–7.5, and 10–12, respectively, for the three ortho- and pyrophosphate anions.

11.4.2 Salts in Processed Dairy Foods

Salts are used extensively in processed cheeses and imitation cheeses to promote a uniform, smooth texture. These additives are sometimes referred to as emulsifying salts because of their ability to aid in dispersion of fat. Although the emulsifying mechanism remains somewhat less than fully defined, anions from the salts when added to processed cheese combine with and remove calcium from the *para*-casein complex and this causes rearrangement and exposure of both polar and nonpolar regions of the cheese proteins. It is also believed that the anions of these salts participate in ionic bridges between protein molecules, and thereby provide a stabilized matrix that entraps the fat in processed cheese [48]. Salts used for cheese processing include mono-, di-, and trisodium phosphate, dipotassium phosphate, sodium hexametaphosphate, sodium acid pyrophosphate, tetrasodium pyrophosphate, sodium aluminum phosphate, and other condensed phosphates, trisodium citrate, tripotassium citrate, sodium tartrate, and sodium potassium tartrate.

The addition of certain phosphates, such as trisodium phosphate, to evaporated milk prevents separation of the butterfat and aqueous phases. The amount required varies with the season of the year and the source of milk. Concentrated milk that is sterilized by a high temperature short-time method frequently gels upon storage. The addition of polyphosphates, such as sodium hexametaphosphate and sodium tripolyphosphate, prevents gel formation through a protein denaturation and solubilization mechanism involving complexation of calcium and magnesium by phosphates.

11.4.3 Phosphates and Water Binding in Animal Tissues

The addition of appropriate phosphates increases the water-holding capacity of raw and cooked meats [27], and these phosphates are used in the production of sausages, in the curing of ham, to decrease drip-losses in poultry and seafoods, and more recently to provide succulence to prepackaged fresh pork and beef. Sodium tripolyphosphate ($Na_5P_3P_{10}$) is the most common phosphate added to processed meat, poultry, and seafoods. It is often used in blends with sodium hexametaphosphate [$(Na\ PO_3)_n$, $n = 10–15$] to increase tolerance to calcium ions that exist in brines used in meat curing. Ortho- and pyrophosphates often precipitate if used in brines containing substantial amounts of calcium, but calcium can be complexed with hexametaphosphate, which remains soluble and prevents calcium phosphate precipitation.

The mechanism by which alkaline phosphates and polyphosphates enhance meat hydration is not clearly understood despite extensive studies. The action may involve the influence of pH changes (Chapter 16), effects of ionic strength, and specific interactions of phosphate anions with divalent cations and myofibrillar proteins. Many believe that calcium complexing and a resulting loosening of the tissue structure is a major function of polyphosphates. It is also believed that binding of polyphosphate anions to proteins and simultaneous cleavage of cross-linkages between actin and myosin results in increased electrostatic repulsion between peptide chains and a swelling of the muscle system. If exterior water is available, it can be then taken up in an immobilized state within the loosened protein network. Further, because the ionic strength has been increased, the interaction between proteins is perhaps reduced to a point where part of the myofibrillar proteins form a colloidal solution. In comminuted meat products, such as bologna and sausage, the addition of sodium chloride (2.5–4.0%) and polyphosphate (0.35–0.5%) contributes to a more stable emulsion, and after cooking to a cohesive network of coagulated proteins.

If the phosphate-induced solubilization occurs primarily on the surface of tissues, as is the case with polyphosphate-dipped (6–12% solution with 0.35–0.5% retention) fish fillets, shellfish, and poultry, a layer of coagulated protein is formed during cooking and this improves moisture retention [38].

11.5 CHELATING AGENTS (SEQUESTRANTS)

Chelating agents or sequestrants play a significant role in food stabilization through reactions with metallic and alkaline earth ions to form complexes that alter the properties of the ions and their effects in foods. Many of the chelating agents employed in the food industry are natural substances, such as polycarboxylic acids (citric, malic, tartaric, oxalic, and succinic), polyphosphoric acids (adenosine triphosphate and pyrophosphate), and macromolecules (porphyrins and proteins). Many metals exist in a naturally chelated state. Examples include magnesium in chlorophyll; copper, zinc, and manganese in various enzymes; iron in proteins such as ferritin; and iron in the porphyrin ring of myoglobin and hemoglobin. When these ions are released by hydrolytic or other degradative reactions, they are free to participate in reactions that lead to discoloration, oxidative rancidity, turbidity, and flavor changes in foods. Chelating agents are sometimes added to form complexes with these metal ions, and thereby stabilize the foods.

Any molecule or ion with an unshared electron pair can coordinate or form complexes with metal ions. Therefore, compounds containing two or more functional groups, such as —OH, —SH,

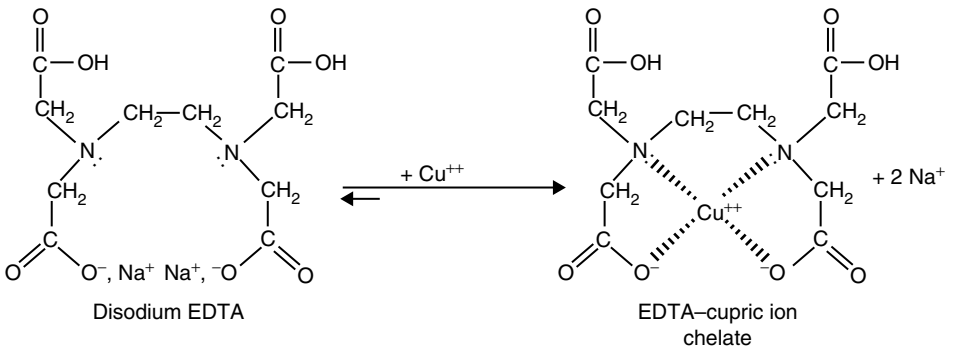


FIGURE 11.6 Schematic representation of chelation of cupric cation (a lipid oxidation catalyst) by disodium EDTA.

—COOH, $-PO_3H_2$, C═O, $-NR_2$, —S—, and —O—, in proper geometrical relation to each other, can chelate metals in a favorable physical environment. Citric acid and its derivatives, various phosphates, and salts of ethylenediaminetetraacetic acid (EDTA) are the most popular chelating agents used in foods. Usually, the ability of a chelating agent (ligand) to form a five- or six-membered ring with a metal is necessary for stable chelation. For example, EDTA forms chelates of high stability with cupric cations because of an initial coordination involving the electron pairs of its nitrogen atoms and the free electron pairs of the anionic oxygen atoms of two of the four carboxyl groups (Figure 11.6). The spatial configuration of the cupric cation–EDTA complex is such that it allows additional coordination of the cupric ion with the free electron pairs of the anionic oxygen atoms of the remaining two carboxyl groups, and this results in an extremely stable complex utilizing all six electron donor groups.

In addition to steric and electronic considerations, factors such as pH influence the formation of strong metal chelates. The nonionized carboxylic acid group is not an efficient donor group, but the carboxylate ion functions effectively. Judicious raising of the pH allows dissociation of the carboxyl group and enhances chelating efficiency. In some instances, hydroxyl ions compete for metal ions and reduce the effectiveness of chelating agents. Metal ions exist in solution as hydrated complexes (metal $\cdot$ H_2O^{M+}), and the rate at which these complexes are disrupted influences the rate at which they can be complexed with chelating agents. The relative attraction of chelating agents for different ions can be determined from stability or equilibrium constants (K = [metal $\cdot$ chelating agent]/[metal][chelating agent]) (Chapter 8). For example, for calcium the stability constant (expressed as log K) is 10.7 with EDTA, 5.0 with pyrophosphate, and 3.5 with citric acid [20]. As the stability constant (K) increases, more of the metal is complexed, leaving less metal in the noncomplexed cation form (i.e., the metal in the complex is more tightly bound).

Chelating agents are not antioxidants in the sense that they arrest oxidation by chain termination or serve as oxygen scavengers. They are, however, valuable antioxidant synergists since they remove metal ions that catalyze oxidation (Chapter 4). When selecting a chelating agent for an antioxidant synergist role, its solubility must be considered. Citric acid and citrate esters (20–200 ppm) in propylene glycol solution are solubilized by fats and oils and thus are effective synergists in all-lipid systems. On the other hand, Na_2EDTA and Na_2Ca–EDTA dissolve to only a limited extent, and are not effective in pure fat systems. The EDTA salts (to 500 ppm), however, are very effective antioxidants in emulsion systems, such as salad dressings, mayonnaise, and margarine, because they can function in the aqueous phase, and especially at the interface between the aqueous and fat phases.

Polyphosphates and EDTA are used in canned seafoods to prevent the formation of glassy crystals of struvite or magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$). Seafoods contain substantial amounts of magnesium ions that sometimes react with ammonium phosphate during storage of canned seafoods to give crystals that may be mistaken as glass contamination. Chelating agents

complex magnesium and minimize struvite formation. Chelating agents also can be used to complex iron, copper, and zinc in seafoods to prevent reactions, particularly with sulfides, that lead to product discoloration. The addition of chelating agents to vegetables prior to blanching can inhibit metal-induced discolorations, and can remove calcium from pectic substances in cell walls and thereby promote tenderness.

Although citric and phosphoric acids are employed as acidulants in soft drink beverages, they also chelate metals that otherwise could promote oxidation of flavor compounds, such as terpenes, and catalyze discoloration reactions. Chelating agents also stabilize fermented malt beverages by complexing copper. Free copper catalyzes oxidation of polyphenolic compounds that subsequently interact with proteins to form permanent hazes or turbidity.

The extremely efficient chelating abilities of some agents, notably synthetic EDTA and natural phytic acid (hexaphosphoinositol), has caused speculation that excessive usage or occurrence in foods could lead to the depletion of calcium and other cationic minerals in the body. To deal with this concern, levels and applications of EDTA are regulated, and in some instances calcium is added to food systems through the use of the Na_2Ca salt of EDTA rather than the all-sodium (Na, Na_2, Na_3, or Na_4 EDTA) or acid forms. However, most current scientific thinking embraces the view that there is little dietary concern about these chelators in the amounts permitted or encountered considering the natural concentrations of calcium and other divalent cations that are present in foods.

11.6 ANTIOXIDANTS

Oxidation occurs when electrons are removed from an atom or group of atoms. Simultaneously, there is a corresponding reduction reaction that involves the addition of electrons to a different atom or group of atoms. Oxidation reactions may or may not involve the addition of oxygen atoms or the removal of hydrogen atoms from the substance being oxidized. Oxidation–reduction reactions are common in biological systems and are also common in foods. Although some oxidation reactions are beneficial in foods, others can lead to detrimental effects including degradation of lipids (Chapter 4), vitamins (Chapter 7), and pigments (Chapter 9) with loss of nutritional value and development of off-flavors. Control of undesirable oxidation reactions in foods is usually achieved by employing processing and packaging techniques that exclude oxygen or involve the addition of appropriate chemical agents.

Before the development of specific chemical technology for the control of free-radical mediated lipid oxidation, the term antioxidant was applied to all substances that inhibited oxidation reactions regardless of the mechanism. For example, ascorbic acid was considered an antioxidant, and was employed to prevent enzymic browning of the cut surfaces of fruits and vegetables (Chapter 6). In this application, ascorbic acid functions as a reducing agent by transferring hydrogen atoms back to quinones that are formed by enzymic oxidation of phenolic compounds. In closed systems, ascorbic acid reacts readily with oxygen and thereby serves as an oxygen scavenger. Likewise, sulfites are readily oxidized in food systems to sulfonates and sulfates, and thereby function as effective antioxidants in foods such as dried fruits (Section 11.7.1). The most commonly employed food antioxidants are phenolic substances. More recently, the term "food antioxidants" often has been applied to those compounds that interrupt the free-radical chain reaction involved in lipid oxidation and those that scavenge singlet oxygen; however, the term probably should not be used in such a narrow sense.

Antioxidants often exhibit variable degrees of efficiency in protecting food systems, and combinations often provide greater overall protection than can be accounted for through the simple additive effects [55]. Thus, mixed antioxidants sometimes have a synergistic action, the mechanisms for which are not completely understood. It is believed, for example, that ascorbic acid can regenerate phenolic antioxidants by supplying hydrogen atoms to the phenoxy radicals that form when the phenolic antioxidants yield hydrogen atoms to the lipid oxidation chain reaction. In order to achieve

this action in lipids, ascorbic acid must be made less polar so it will dissolve in fat. This is done by esterification to fatty acids to form compounds, such as ascorbyl palmitate.

The presence of transition-state metal ions, particularly copper and iron, promotes lipid oxidation through catalytic actions (Chapters 4 and 8). These metallic pro-oxidants are frequently inactivated by adding chelating agents, such as citric acid or EDTA (Section 11.5). In this role chelating agents are also referred to as synergists since they greatly enhance the action of the phenolic antioxidants. However, they are often ineffective as antioxidants when employed alone.

Many naturally occurring substances possess antioxidant capabilities, and the tocopherols are noted examples that are widely employed (Chapter 4). Recently, extractives of spices containing polyphenolic substances, particularly from rosemary, also have been successfully commercially exploited as natural antioxidants. Gossypol that occurs naturally in cottonseed is an antioxidant, but it has toxic properties. Other naturally occurring antioxidants are coniferyl alcohol (found in plants) and guiaconic and guiacic acid (from gum guaiac). All of these are structurally related to butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and di-*t*-butylhydroquinone (TBHQ), which are synthetic phenolic antioxidants currently approved for use in foods (Chapter 4). Nordihydroguairetic acid, a compound related to some of the constituents of gum guaiac, is an effective antioxidant, but its use directly in foods has been suspended because of toxic effects. All of these phenolic substances serve as oxidation terminators by participating in the reactions through resonance stabilized free-radical forms [55], but they also are believed to serve as singlet oxygen scavengers. On the other hand, β-carotene is considered to function more efficiently as a singlet oxygen scavenger than phenolic substances.

Thiodipropionic acid and dilauryl thiodipropionate remain as approved food antioxidants, even though removal of these compounds from the approved list has been proposed because they were not being used in foods. The presence of a sulfur atom in the thiodipropionates has led to speculation that they could cause off-flavors, but this view is unfounded. A more compelling reason that the thiodipropionates have not been used in foods is their failure to inhibit lipid oxidation in foods, as measured by peroxide value, when used at permitted levels (to 200 ppm) [32]. The classic role of thiodipropionates is as secondary antioxidants, where at high concentrations (>1000 ppm), they degrade hydroperoxides formed during olefin oxidation to relatively stable end products, and used as such, they are useful in stabilizing synthetic polyolefins.

Although thiodipropionates, at levels allowed in foods, are ineffective in reducing peroxide values, they are highly effective in decomposing peracids (Figure 11.7) found in oxidizing lipids [32]. Peracids are very efficient substances for mediating the oxidation of double bonds to epoxides, and in the presence of water, epoxides formed by this reaction readily hydrolyze to form diols. When these reactions occur with cholesterol, both cholesterol epoxide and the cholesterol–triol derivative are formed, and these cholesterol oxides are widely considered potentially mutagenic and atherogenic, respectively, to humans [42]. Because the thiodipropionates readily inhibit the accumulation of peracids, they have been retained as approved antioxidants by the U.S. Food and Drug Administration (FDA).

A chemical structure similar to that in the thiodipropionates occurs in methionine (Chapter 5), and accounts, presumably by an analogous mechanism, for some of the antioxidant properties shown by

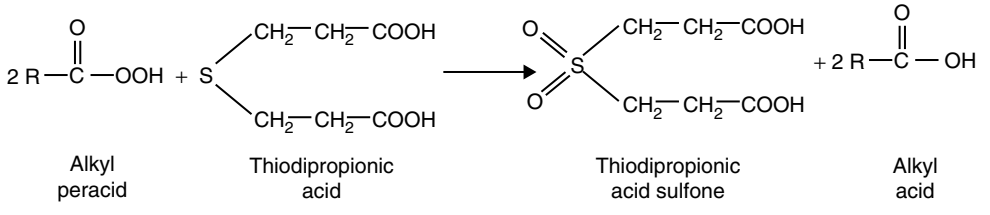


FIGURE 11.7 Mechanism for peracid destruction in oxidizing lipids by thiodipropionic acid.

proteins. Reaction of a sulfide (i.e., thioether) with one peracid or hydroperoxide yields a sulfoxide while reactions with two peracids or hydroperoxides yields a sulfone.

11.7 ANTIMICROBIAL AGENTS

Chemical preservatives with antimicrobial properties play an important role in preventing spoilage and assuring safety of many foods. Some of these are discussed in the following section.

11.7.1 Sulfites and Sulfur Dioxide

Sulfur dioxide (SO_2) and its derivatives have long been used in foods as general food preservatives. They are added to food to inhibit nonenzymic browning, to inhibit enzyme catalyzed reactions, to inhibit and control microorganisms, and to act as an antioxidant and a reducing agent. Generally, SO_2 and its derivatives are metabolized to sulfate and excreted in the urine without any obvious pathologic results [60]. However, because of somewhat recently recognized severe reactions to sulfur dioxide and its derivatives by some sensitive asthmatics, their use in foods is currently regulated and subject to rigorous labeling restrictions. Nonetheless, these preservatives serve key roles in contemporary foods.

The commonly used forms in foods include SO_2 gas, and the sodium, potassium, or calcium salts of sulfite (SO_3^{2-}), bisulfite (HSO_3^-), or metabisulfite ($S_2O_5^{2-}$). The most frequently used sulfiting agents are the sodium and potassium metabisulfites because they exhibit good stability toward autoxidation in the solid phase. However, gaseous sulfur dioxide is employed where leaching of solids causes problems or where the gas may also serve as an acid for the control of pH.

Although the traditional names for the anions of these salts are still widely used (sulfites, bisulfites, and metabisulfites), they have been designated by IUPAC as the sulfur (IV) oxoanions, sulfites (SO_3^{2-}), hydrogensulfites (HSO_3^-), and disulfites ($S_2O_5^{2-}$), respectively. The oxoacids, H_2SO_3 and $H_2S_3O_5$, are designated as sulfurous and disulfurous acids, respectively [60].

Widely held views also have changed somewhat on the existence of sulfurous acid in aqueous solutions. Earlier, it was presumed that when sulfur dioxide was dissolved in water, it formed sulfurous acid, because the salts of simple oxoanions of sulfur (IV) (valence +4) are salts of this acid (H_2SO_3; sulfurous acid). However, evidence for the existence of free sulfurous acid has not been found, and it has been estimated that it accounts for less than 3% of nondissociated dissolved SO_2. Instead, solution of SO_2 yields only weak interactions with water that results in a nondissociated complex which is particularly abundant below pH 2. This complex has been denoted $SO_2 \cdot H_2O$, and a distinction between this complex and sulfurous acid is not generally made [60] (Equation 11.12).

Since the acidity of solutions of SO_2 is significant, and free sulfurous acid is not found, it is argued that the strong acid, $HSO_2(OH)$, exists in small amounts, and its dissociation leads predominantly to the HSO_3^- (bisulfite) ion rather than the $SO_2(OH)^-$ ion that has been estimated to occur to an extent of only 2.5% of the hydrogen sulfite species (Equation 11.13). The HSO_3^- ion predominates from pH 3 to 7, but above pH 7, the SO_3^{2-} ion is most abundant (Equation 11.14). The pK_a for the first dissociation of "sulfurous acid" is 1.86, and the second dissociation has a pK_a of 7.18. In dilute solutions of HSO_3^- (10^{-2} M), little metabisulfite (disulfite) ion exists, but as the concentration of bisulfite increases, the proportion increases rapidly (Equation 11.15). Thus, the relative proportion of each form depends on the pH of the solution, the ionic strength of the sulfur (IV) oxospecies, and the concentration of neutral salts [60].

$$SO_2 + H_2O \rightleftarrows SO_2 \cdot H_2O \tag{11.12}$$

$$SO_2 \cdot H_2O \rightleftarrows HSO_2(OH) \rightleftarrows HSO_3^- + H^+ \tag{11.13}$$

$$HSO_3^- \rightleftharpoons H^+ + SO_3^{2-} \tag{11.14}$$

$$2HSO_3^- \rightleftharpoons S_2O_5^{2-} + H_2O \tag{11.15}$$

Sulfur dioxide is most effective as an antimicrobial agent in acid media, and this effect may result from conditions that permit undissociated compounds to penetrate the cell wall. At high pH, it has been noted that the HSO_3^- ion is effective against bacteria, but not against yeasts. Sulfur dioxide acts as both a biocidal and biostatic agent, and is more active against bacteria than molds and yeasts. Also, it is more effective against Gram-negative bacteria than Gram-positive bacteria.

The nucleophilicity of the sulfite ion is believed responsible for much of the effectiveness of sulfur dioxide as a food preservative in both microbial and chemical applications [61]. Some evidence has accumulated that the interaction of sulfur (IV) oxospecies with nucleic acids cause the biostatic and biocidal effects [60]. Other postulated mechanisms by which sulfur (IV) oxospecies inhibit microorganisms include the reaction of bisulfite with acetaldehyde in the cell, the reduction of essential disulfide linkages in enzymes, and the formation of bisulfite addition compounds that interfere with respiratory reactions involving nicotinamide dinucleotide.

Of the known inhibitors of nonenzymic browning in foods (Chapter 3), sulfur dioxide is one of the most effective. Multiple chemical mechanisms are involved in sulfur dioxide inhibition of nonenzymic browning (Figure 11.8), but one of the most important involves the reaction of sulfur (IV) oxoanions (bisulfite) with carbonyl groups of reducing sugars and other compounds participating in browning. These reversible bisulfite addition compounds, thus, bind carbonyl groups to retard the browning process, but it also has been proposed that the reaction removes carbonyl chromophores in melanoidin structures that leads to a bleaching effect on the pigment. Sulfur (IV) oxoanions also irreversibly react with hydroxyl groups, especially those in the 4-position, on sugar and ascorbic acid intermediates in browning reactions to yield sulfonates ($R{-}CHSO_3^-{-}CH_2{-}R'$). The formation of relatively stable sulfonate derivatives retards the overall reaction and interferes with pathways that are particularly prone to producing colored pigments [60].

Sulfur dioxide also inhibits certain enzyme-catalyzed reactions, notably enzymic browning, that are important in food preservation. The production of brown pigments by enzyme-catalyzed oxidation of phenolic compounds can lead to a serious quality problem during the handling of some fresh fruits and vegetables (Chapter 6). However, the use of sulfite or metabisulfite sprays or dips with or without added citric acid provides effective control of enzymic browning in prepeeled and presliced potatoes, carrots, and apples.

Sulfur dioxide also functions as an antioxidant in a variety of food systems, but it is not usually employed for this purpose. When it is added to beer, the development of oxidized flavors is inhibited significantly during storage. The red color of fresh meat also can be effectively maintained by the presence of sulfur dioxide. However, this practice is not permitted because of the potential for masking deterioration in abused meat products.

When added during manufacture of wheat flour doughs, sulfur dioxide effects a reversible cleavage of protein disulfide bonds. In the instance of cookie manufacture, the addition of sodium bisulfite reduces mixing time and the elasticity of the dough that facilitates dough sheeting, and it also reduces variations caused by different lots of flour [60]. Before drying of fruits, gaseous sulfur dioxide is often applied, and this is sometimes done in the presence of buffering agents (i.e., $NaHCO_3$). This treatment prevents browning and induces a reversible bleaching of anthocyanin pigments. The resulting properties are desired in products, such as those used to make white wines and maraschino cherries. Levels of sulfur dioxide encountered in dried fruits immediately following processing sometimes approach 2000 ppm. However, much lower amounts are found in most other foods because concentrations above 500 ppm give noticeably disagreeable flavors, and because sulfites tend to volatilize and/or react during storage and cooking.

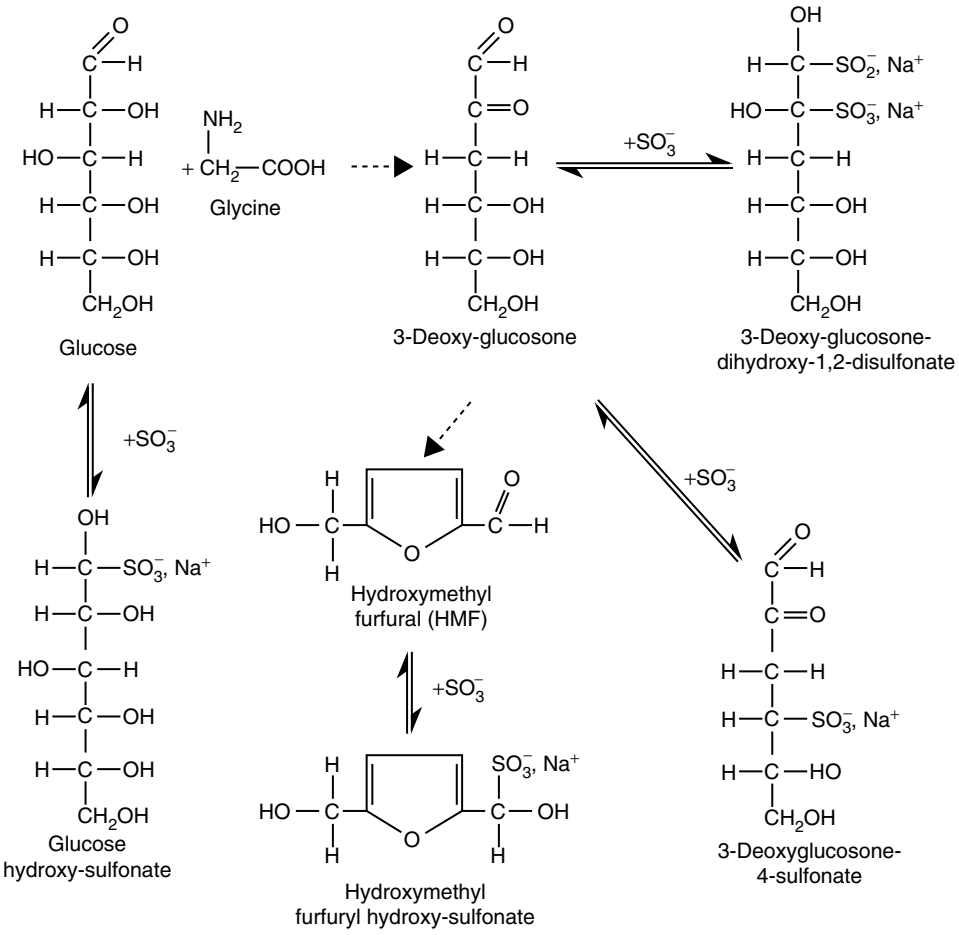


FIGURE 11.8 Mechanisms of inhibition of Maillard (carbonyl-amino) browning by some sulfur (IV) oxoanions (bisulfite, sulfite).

11.7.2 Nitrite and Nitrate Salts

The potassium and sodium salts of nitrite and nitrate are commonly used in curing mixtures for meats to develop and fix the color, to inhibit microorganisms, and to develop characteristic flavors [50]. Nitrite rather than nitrate is apparently the functional constituent. Nitrites in meat form nitric oxide, which reacts with heme compounds to form nitrosomyoglobin, the pigment responsible for the pink color of cured meats (Chapter 9). Sensory evaluations also indicate that nitrite contributes to cured meat flavor apparently through an antioxidant role, but the details of this chemistry are poorly understood [46]. Furthermore, nitrites (150–200 ppm) inhibit clostridia in canned-comminuted and cured meats. In this regard, nitrite is more effective at pH 5.0–5.5 than it is at higher pH values. The antimicrobial mechanism of nitrite is unknown, but it has been suggested that nitrite reacts with sulfhydryl groups to create compounds that are not metabolized by microorganisms under anaerobic conditions.

Nitrites have been shown to be involved in the formation of low, but possibly toxic levels, of nitrosamines in certain cured meats. The chemistry and health implications of nitrosamines are discussed in Chapter 12. Nitrate salts also occur naturally in many foods, including vegetables such as spinach. The accumulation of large amounts of nitrate in plant tissues grown on heavily fertilized soils is of concern, particularly in infant foods prepared from these tissues. The reduction of nitrate

to nitrite in the intestine, with subsequent absorption, could lead to cyanosis due to methmyoglobin formation. For these reasons, the use of nitrites and nitrates in foods has been questioned. The antimicrobial capability of nitrite provides some justification for its use in cured meats, especially where growth of *C. botulinum* is possible. However, in preserved products where botulism does not present a hazard, there appears to be little justification for adding nitrates and nitrites.

11.7.3 Acetic Acid

The preservation of foods with acetic acid (CH_3COOH) in the form of vinegar dates to antiquity. In addition to vinegar (4% acetic acid) and acetic acid, also used in food are sodium acetate (CH_3COONa), potassium acetate (CH_3COOK), calcium acetate [$(CH_3{-}COO)_2Ca$], and sodium diacetate ($CH_3{-}COONa \cdot CH_3{-}COOH \cdot \frac{1}{2}H_2O$). The salts are used in bread and other baked goods (0.1–0.4%) to prevent ropiness and the growth of molds without interfering with yeast [8]. Vinegar and acetic acid are used in pickled meats and fish products. If fermentable carbohydrates are present, at least 3.6% acid must be present to prevent growth of lactic acid bacilli and yeasts. Acetic acid is also used in foods such as catsup, mayonnaise, and pickles where it serves a dual function of inhibiting microorganisms and contributing to flavor. The antimicrobial activity of acetic acid increases as the pH is decreased, a property analogous to that found for other aliphatic fatty acids.

11.7.4 Propionic Acid

Propionic acid ($CH_3{-}CH_2{-}COOH$) and its sodium and calcium salts exert antimicrobial activity against molds and a few bacteria. This compound occurs naturally in Swiss cheese (up to l% by weight), where it is produced by *Propionibacterium shermanii* [8]. Propionic acid has found extensive use in the bakery field where it not only inhibits molds effectively, but also is active against the ropy bread organism, *Bacillus mesentericus*. Levels of use generally range up to 0.3% by weight. As with other carboxylic acid antimicrobial agents, the undissociated form of propionic acid is active, and the range of effectiveness extends up to pH 5.0 in most applications. The toxicity of propionic acid to molds and certain bacteria is related to the inability of the affected organisms to metabolize the three carbon skeleton. In mammals, propionic acid is metabolized in a manner similar to that of other fatty acids, and it has not been shown to cause any toxic effects at the levels encountered in foods.

11.7.5 Sorbic Acid and Other Medium Chain Fatty Acids

Medium chain and longer, monocarboxylic, aliphatic fatty acids exhibit antimicrobial, especially antimycotic, activities. The α-unsaturated fatty acid analogs are especially effective. Sorbic acid ($C{-}C{=}C{-}C{=}C{-}COOH$) and its sodium and potassium salts are widely used to inhibit mold and yeasts in a wide variety of foods including cheese, baked products, fruit juices, wine, and pickles. Sorbic acid is found in nature, most notably in the berries of the mountain ash. Commercial sorbate salts employed in the food industry are synthesized industrially, and are comprised largely of *trans* fatty acid isomers. Since sorbic acid is chemically a fatty substance, from a labeling perspective, its use contributes to the *trans* fat content of a food.

Sorbic acid is particularly effective in preventing mold growth, and it contributes little flavor at the concentrations employed (up to 0.3% by weight). The method of application may involve direct incorporation, surface coatings, or incorporation in a wrapping material. The activity of sorbic acid increases as the pH decreases, indicating that the undissociated form is more inhibitory than the dissociated form. In general, sorbic acid is effective up to pH 6.5, which is considerably above the effective pH ranges for propionic and benzoic acids.

The antimycotic action of sorbic acid appears to arise because molds are unable to metabolize the α-unsaturated diene system of its aliphatic chain. It has been suggested that the diene structure

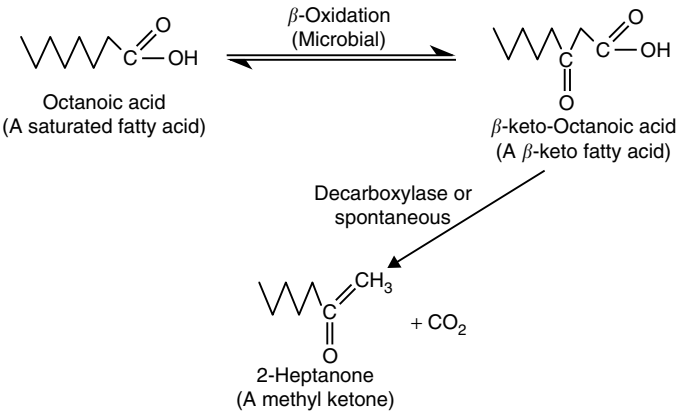


FIGURE 11.9 Mold-mediated detoxification of a fatty acid (octanoic acid) via β-oxidation followed by decarboxylation to yield a methyl ketone (heptanone).

of sorbic acid interferes with cellular dehydrogenases that normally dehydrogenate fatty acids as the first step in oxidation.

Saturated fatty acids (C_2—C_{12}) are also moderately inhibitory to many molds, such as *Penicillium roqueforti*. However, some of these molds are capable of mediating β-oxidation of saturated fatty acids to corresponding β-keto acids, especially when the concentration of the acid is only marginally inhibitory. Decarboxylation of the resulting β-keto acid yields the corresponding methyl ketone (Figure 11.9) which does not exhibit antimicrobial properties. On the other hand, this mechanism for the formation of methyl ketones accounts for a major portion of the characteristic flavor of mold-ripened blue cheeses. A few molds have also been shown to metabolize sorbic acid, and it has been suggested that this metabolism proceeds through β-oxidation, similar to that in mammals. All evidence indicates that animals and humans metabolize sorbic acid in much the same way as they do other naturally occurring fatty acids.

Although sorbic acid might at first appear quite stable and unreactive, it is quite often microbiologically or chemically altered in foods. Two other mechanisms for deactivating the antimicrobial properties of sorbic acid are shown in Figure 11.10. The reaction labeled "a" in Figure 11.10 has been demonstrated in molds, especially *P. roqueforti*. This involves direct decarboxylation of sorbic acid to yield the hydrocarbon, 1,3-pentadiene. The intense aroma of this compound can cause gasoline or hydrocarbon-like off-flavors when mold growth occurs in foods containing sorbic acid, especially on the surface of cheese treated with sorbate.

If wine containing sorbic acid undergoes spoilage in the bottle by lactic acid bacteria, an off-flavor described as geranium-like develops [11]. Lactic acid bacteria reduce sorbic acid to sorbyl alcohol, and then, because of the acid conditions they have created, cause a rearrangement to a secondary alcohol (Figure 11.10b). The final reaction involves the formation of an ethoxylated hexadiene that has a pronounced, easily recognized aroma of geranium leaves.

Sorbic acid is sometimes used in combination with sulfur dioxide, and this leads to reactions that deplete both sorbic acid and sulfur (IV) oxoanions (Figure 11.11) [23]. Under aerobic conditions, especially in the presence of light, SO_3^- radicals are formed, and these radicals sulfonate olefin bonds as well as promote oxidation of sorbic acid. This reaction, uniquely involving sorbic acid, is not noticeably affected by the presence of conventional antioxidants, and aerobically held foods containing sulfur dioxide and sorbic acid are very susceptible to autoxidation. Under anaerobic conditions, the combination of sorbic acid and sulfur dioxide in foods results in a much slower nucleophilic reaction of the sulfite ion (SO_3^-) with the diene (1,4-addition) in sorbic acid to yield 5-sulfo-3-hexenoic acid (Figure 11.11).

Reactions between sorbic acid and proteins occur when sorbic acid is used in certain foods, such as wheat dough, whose proteins contain substantial amounts of oxidized or reduced thiol groups

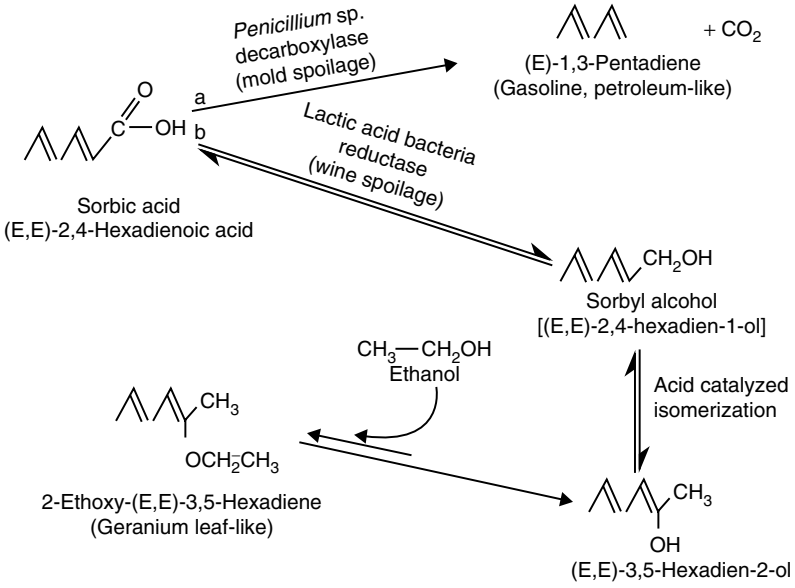


FIGURE 11.10 Enzymic conversions destroying the antimicrobial properties of sorbic acid: (a) decarboxylation carried out by *Penicillium* sp.; (b) formation of ethoxylated diene hydrocarbon in wine resulting from a reduction of the carboxyl group followed by rearrangement and development of an ether.

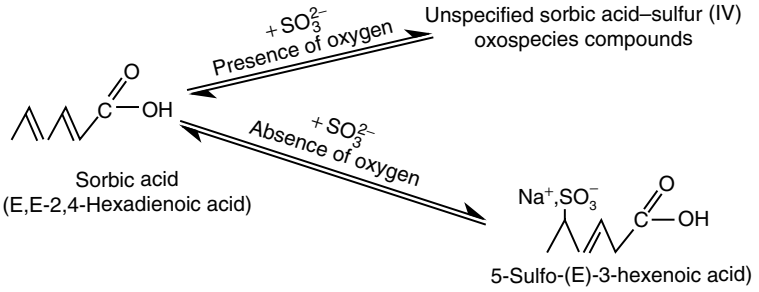


FIGURE 11.11 Reactions of sorbic acid with sulfur dioxide [sulfur (IV) anions].

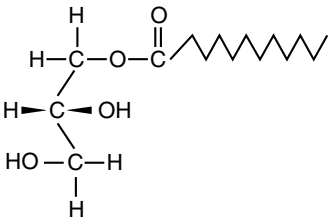
(R—S—S—R in cystine and R—SH in cysteine, respectively). The thiol groups (R—SH) dissociate to thiolate ions ($R—S^-$) that are reactive nucleophiles, and they react mainly by 1,6-addition to the conjugated diene of sorbic acid. This reaction, which readily occurs at higher pH (>5) and elevated temperatures, binds the protein to the sorbic acid (e.g., during bread baking). Although the reaction is reversible under very acidic conditions (pH < 1), the usual consequence at the higher pH values of foods is the loss of the preservative action of sorbic acid [33].

While sorbic acid and potassium sorbate have gained wide recognition as antimycotics, more recent research has established that sorbate has broad antimicrobial activity that extends to yeasts and many bacterial species that are involved in spoilage of fresh poultry, fish, and meats. It is especially effective in retarding toxigenesis of *C. botulinum* in bacon and refrigerated fresh fish packaged in modified atmospheres.

11.7.6 Glyceryl Esters

Many fatty acids and monoglycerides show pronounced antimicrobial activity against Gram-positive bacteria and some yeasts [31]. Unsaturated members, especially those with 18-carbon atoms, show

strong activity as fatty acids; the medium chain length members (12-carbon atoms) are most inhibitory when esterified to glycerol. Glyceryl monolaurate (I), also known under the tradename of Monolaurin, is inhibitory against several potentially pathogenic staphylococcus and streptococcus when present at concentrations of 15–250 ppm. It is commonly used in cosmetics, and because of its lipid nature can be used in some foods.

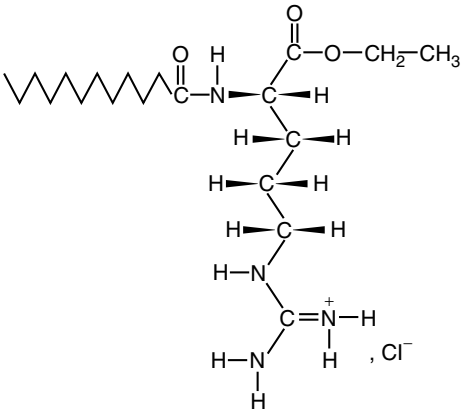


Monolauren (I)

Lipophilic agents of this kind also exhibit inhibitory activity against *C. botulinum*, and glyceryl monolaurate, serving this function, may find applications in cured meats and in refrigerated, packaged fresh fish. The inhibitory effect of lipophilic glyceride derivatives apparently relates to their ability to facilitate the conduction of protons through the cell membranes that effectively destroys the proton-motive force that is needed for substrate transport [18]. Cell-killing effects are observed only at high concentrations of the compounds, and death apparently results from the generation of holes in cell membranes.

11.7.7 Lauric Arginate

Lauric arginate (ethyl-*N*-dodecanoyl-L-arginate hydrochloride; II) is a newly available antimicrobial that, like glyceryl monolaurate, contains a medium chain fatty acid moiety.

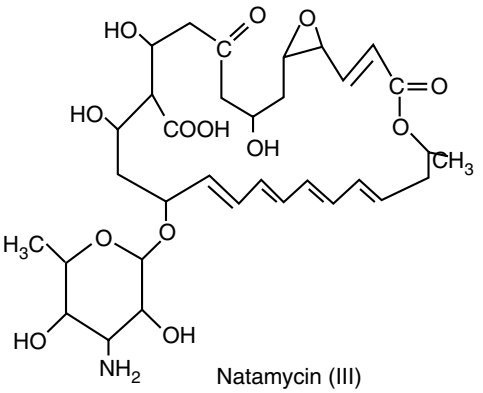


Lauric arginate (II)

Lauric arginate has received U.S. GRAS status approval, and it exhibits broad spectrum antimicrobial efficacy that is anticipated to be useful in the control of food pathogens (*Campylobacter*, *Salmonella*, *Clostridium*, *Escherichia*, and *Staphylococcus*). The mode of action of lauric arginate involves the disruption of the plasma membrane lipid bilayer, which leads to interferences with metabolic processes and cell cycling [3].

11.7.8 Natamycin

Natamycin or pimaricin (CAS Reg. No. 768-93-8) is a polyene macrolide antimycotic (III) that has been approved in the United States for use against molds on cured cheeses.



Natamycin (III)

This mold inhibitor is highly effective when applied to surfaces of foods exposed directly to air where mold has a tendency to proliferate. The mechanism of action of natamycin is believed to result from a binding of natamycin to sterol molecules in fungal cell membranes that alters the permeability of the membranes and thereby causes disruption of cell processes. Natamycin is especially attractive for application on fermented foods, such as cured cheeses, because it selectively inhibits molds while allowing normal growth and metabolism of ripening bacteria.

11.7.9 Benzoic Acid

Benzoic acid (C_6H_5COOH) has been widely employed as an antimicrobial agent in foods [5], and it occurs naturally in cranberries, prunes, cinnamon, and cloves. The undissociated acid is the form with antimicrobial activity, and it exhibits optimum activity in the pH range of 2.5–4.0, making it well suited for use in acid foods such as fruit juices, carbonated beverages, pickles, and sauerkraut. Benzoates exhibit very little antimicrobial activity in foods with pH values above 5.2–5.5. Sodium and potassium salts of benzoic acid are generally used because they are more readily dispersible in aqueous foods than the acid form. Once in a suitably acidic product, much of a benzoate salt converts to the active protonated form that is most effective against yeasts and bacteria and least effective against molds. Often benzoic acid is used in combination with sorbic acid or parabens, and levels of use usually range from 0.05% to 0.1% by weight.

The mode of antimicrobial activity of benzoic acid has not been clearly established. However, the lipophilic character of protonated benzoic acid is believed to facilitate entry of the molecule into cell membranes and interiors. Depending on the microbial type, evidence indicates that multiple modes of activity may be involved, including both disruption of proton-motive forces and inhibition of key metabolic enzymes. Benzoic acid has been found to cause no deleterious effects in humans when used in small amounts. It is readily eliminated from the body primarily after conjugation with glycine (Figure 11.12) to form hippuric acid (benzoyl glycine). This detoxification step precludes accumulation of benzoic acid in the body.

11.7.10 *p*-Hydroxybenzoate Alkyl Esters

The parabens are a group of alkyl esters of *p*-hydroxybenzoic acid that have been used widely as antimicrobial agents in foods, pharmaceutical products, and cosmetics. The methyl (IV), propyl (V),

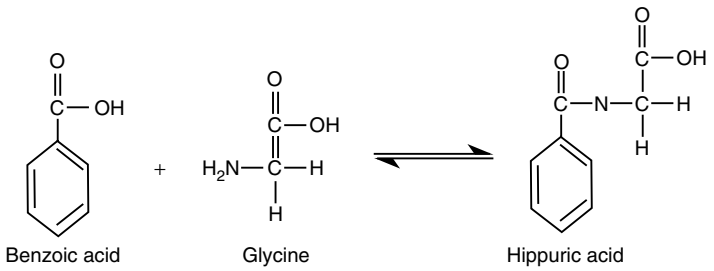
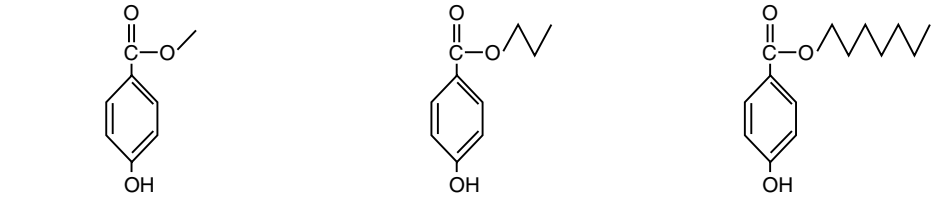


FIGURE 11.12 Metabolic conjugation of the preservative benzoic acid with glycine to facilitate excretion.

and heptyl (VI) esters are used domestically, and in some other countries the ethyl and butyl esters are used as well.



Parabens are used as microbial preservatives in baked goods, soft drinks, beer, olives, pickles, jams, jellies, and syrups. They have little effect on flavor, are effective inhibitors of molds and yeasts (0.05–0.1% by weight), and are relatively ineffective against bacteria, especially Gram-negative bacteria [8]. The antimicrobial activity of parabens increases and their solubility in water decreases with increases in the length of the alkyl chain. The shorter chain members often are used because of their solubility characteristics. In contrast to other antimycotic agents, the parabens are active at pH 7 and higher, apparently because of their ability to remain undissociated at these pH values. The phenolic group provides a weak acid character to the molecule. The ester linkage is stable to hydrolysis even at temperatures used for sterilization. The parabens have many properties in common with benzoic acid and they are often used together. Parabens exhibit a low order of toxicity to humans and are excreted in the urine after hydrolysis of the ester group and subsequent metabolic conjugation.

11.7.11 Epoxides

Most antimicrobial agents used in foods exhibit inhibitory rather than lethal effects at the concentrations employed. However, exceptions occur with ethylene (VII) and propylene (VIII) oxides.

These chemical sterilants are used to treat certain low moisture foods and to sterilize aseptic packaging materials. In order to achieve intimate contact with microorganisms the epoxides are used in a vapor state, and after adequate exposure, most of the residual unreacted epoxide is removed by flushing and evacuation.

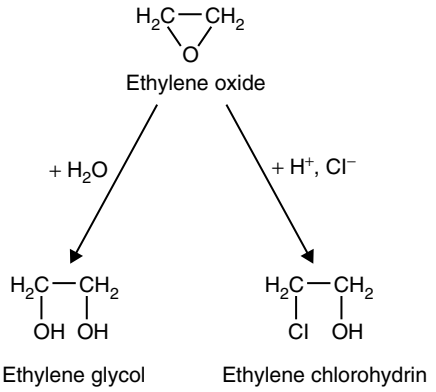


FIGURE 11.13 Reactions of ethylene oxide with water and chloride ion, respectively.

The epoxides are reactive cyclic ethers that destroy all forms of microorganisms, including spores and even viruses, but the mechanism of action of epoxides is poorly understood. In the case of ethylene oxide, it has been proposed that alkylation of essential intermediary metabolites having a hydroethyl group ($-CH_2-CH_2-OH$) could account for the lethal results [8]. The site of attack would be any labile hydrogen in the metabolic system. The epoxides also react with water to form corresponding glycols (Figure 11.13). However, the toxicity of the glycols is low, and therefore, can not account for the inhibitory effect.

Since the majority of the active epoxide is removed from the treated food, and the glycols formed are of low toxicity, it might appear that these gaseous sterilants would be used extensively. Their use, however, is limited to dry items, such as nutmeats and spices, because reaction with water rapidly depletes the concentration of epoxides in high-moisture foods. Spices often contain high microbial loads and are destined for incorporation into perishable foods. Thermal sterilization of spices is unsuitable because important flavor compounds are volatile and the product is generally unstable to heat. Thus, treatment with epoxides is a suitable method for reducing the microbial load.

The potential formation of relatively toxic chlorohydrins as a result of reactions between epoxides and inorganic chlorides (Figure 11.13) is a point of some concern. However, there are reports that dietary chlorohydrin in low concentrations causes no ill effect [62]. Another consideration in the use of epoxides is their possible adverse effects on vitamins, including riboflavin, niacin, and pyridoxine.

Ethylene oxide (boiling point, 13.2°C) is more reactive than propylene oxide and is also more volatile and flammable. For safety purposes, ethylene oxide is often supplied as a mixture consisting of l0% ethylene oxide and 90% carbon dioxide. The product to be sterilized is placed in a closed chamber, the chamber is evacuated, then pressurized to 30 lbs with the ethylene oxide–carbon dioxide mixture. This pressure is needed to provide a concentration of epoxide sufficient to kill microorganisms in a reasonable time. When propylene oxide (boiling point, 34.3°C) is used, sufficient heat must be applied to maintain the epoxide in a gaseous state.

11.7.12 Antibiotics

Antibiotics comprise a large group of antimicrobial agents produced naturally by a variety of microorganisms. They exhibit selective antimicrobial activity, and their applications in medicine have contributed significantly to the field of chemotherapy. The successes of antibiotics in controlling pathogenic microorganisms in living animals have led to extensive investigations into their potential applications in food preservation. However, because of the fear that routine use of antibiotics will cause resistant organisms to evolve, their application to foods, with one exception (nisin), is not currently permitted in the United States. The development of resistant strains of organisms

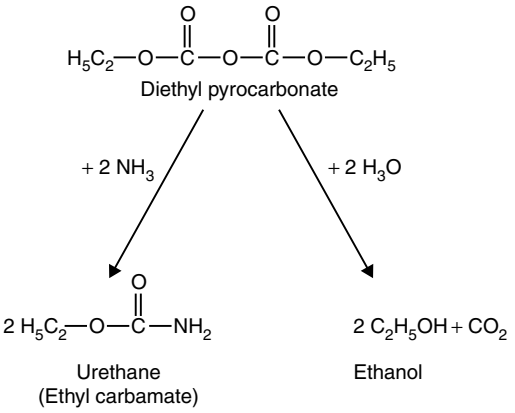


FIGURE 11.14 Reactions showing the hydrolysis and amidization of diethyl pyrocarbonate.

would be of particular concern if an antibiotic proposed for use in food is also used in a medical application.

Nisin, a polypeptide antibiotic, is produced by lactic streptococci, and in the United States, it is now permitted in high-moisture processed cheese products where it is used to prevent potential outgrowth of *C. botulinum*. Nisin has been explored extensively for applications in food preservation. It is active against Gram-positive organisms, especially in preventing the outgrowth of spores [49], and it is not used in medical applications. Nisin is also used in other parts of the world for prevention of spoilage of dairy products, such as processed cheese and condensed milk. Nisin is not effective against Gram-negative spoilage organisms, and some strains of clostridia are resistant. However, nisin is essentially nontoxic to humans, does not lead to cross-resistance with medical antibiotics, and is degraded harmlessly in the intestinal tract.

Some other countries allow limited use of relatively few other antibiotics. These include chlortetracycline and oxytetracycline [8]. Most actual or proposed applications of antibiotics in foods involve their use as adjuncts to other methods of food preservation. Notably, this includes delaying spoilage of refrigerated, perishable foods, and reducing the severity of thermal processes. Fresh meats, fish, and poultry comprise a group of perishable products that could benefit from the action of broad spectrum antibiotics. In fact, many years ago, the U.S. FDA permitted dipping whole poultry carcasses into solutions of chlortetracycline or oxytetracycline. This increased the shelf life of the poultry, and residual antibiotics were destroyed by usual cooking methods.

The biochemical modes of actions for antibiotics are just coming into focus, with research efforts emphasizing molecular mechanisms. In addition, there is a continuing search for natural preservatives that hopefully will be suitable for application to foods. However, the necessarily stringent requirements placed on substances for food applications indicate that acceptable substances will be difficult to find.

11.7.13 Diethyl Pyrocarbonate

Diethyl pyrocarbonate has been used as an antimicrobial food additive for beverages such as fruit juices, wine, and beer. The advantage of diethyl pyrocarbonate is that it can be used in a cold pasteurization process for aqueous solutions, following which it readily hydrolyzes to ethanol and carbon dioxide (Figure 11.14). Usage levels between 120 and 300 ppm in acid beverages (below pH 4.0) cause complete destruction of yeasts in about 60 min. Other organisms, such as lactic acid bacteria, are more resistant and sterilization is achieved only when the microbial load is low ($<500\ mL^{-1}$) and the pH is below 4.0. The low pH retards the rate of diethyl pyrocarbonate decomposition and intensifies its effectiveness.

TABLE 11.3
Relative Sweetness of Some Intensely Sweet Substances

Substance	Relative Sweetness Values[a] (sucrose = 1, wt. basis)
Acesulfame K	200
Alitame	2,000
Aspartame	180–200
Cyclamate	30
Glycyrrhizin	50–100
Monellin	3,000
Neohesperitin dihydrochalcone	1,600–2,000
Neotame	7,000–13,000
Saccharin	300–400
Stevioside	300
Sucralose	600–800
Thaumatin	1,600–2,000

[a] Commonly cited relative sweetness values are listed; however, the concentration and the food or beverage matrix may greatly influence actual relative sweetness values for sweeteners.

Concentrated diethyl pyrocarbonate is an irritant. However, since hydrolysis is essentially complete within 24 h in acid beverages, there is little concern for direct toxicity. Unfortunately, diethyl pyrocarbonate reacts with a variety of compounds to form carbethoxy derivatives and ethyl esters. Specifically, diethyl pyrocarbonate reacts readily with ammonia to yield urethane (ethyl carbamate; Figure 11.14). Ostensibly this reaction was considered responsible for urethane found in foods treated with diethyl pyrocarbonate, and a ban on the use of diethyl pyrocarbonate was issued in 1972 because urethane is a known carcinogen. Since ammonia is ubiquitous in plant and animal tissues, it seemed reasonable that foods treated with diethyl pyrocarbonate will contain some urethane.

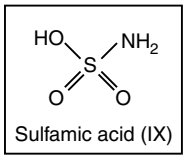
However, it was shown later that urethane occurs intrinsically in fermented foods and beverages, and it is usually present below 10 ppb in most fermented foods, including bread, wine, and beer [26]. It has been suggested that the major pathway for its production in these foods is the reaction of urea, from arginine metabolism, with ethanol. Alcoholic beverages contain much higher levels of urethane than nonalcoholic foods, and levels up to 10 ppm have been reported in stone fruit brandies. In spite of these natural occurrences of urethane, addition of diethyl pyrocarbonate is no longer permitted in foods in the United States because of the potential for elevating levels of a carcinogen in foods.

11.8 INTENSELY SWEET NON-NUTRITIVE AND LOW-CALORIE SWEETENERS

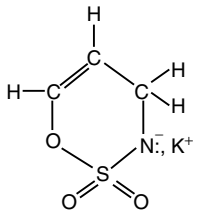
Non-nutritive and low-calorie sweeteners encompass a broad group of substances that evoke a sweet taste or enhance the perception of sweet tastes (see Chapter 10). The ban on the use of cyclamates in the United States along with questions raised about the safety of saccharin initiated an intensive search for alternative low-calorie sweeteners to meet the demand for low-calorie foods and beverages. This has led to the discovery of many new sweet molecules, and the number of viable potentially commercially useful, low-calorie sweeteners continues to grow. The relative sweetness values for some of these substances are given in Table 11.3.

11.8.1 Sulfoamides: Cyclamate, Saccharin, and Acesulfame K

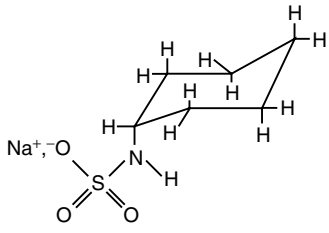
The sulfoamide sweeteners are substances that are structurally related through the sulfamic acid grouping (IX), and commercially available members include acesulfame K (X), cyclamate (XI), and saccharin (XII).



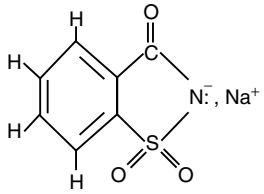
Sulfamic acid (IX)



Acesulfame K (X)



Sodium cyclamate (XI)
(Sodium *N*-cyclohexylsulfamate)

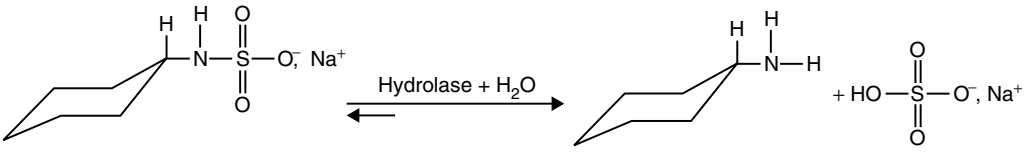


Sodium saccharin (XII)

Cyclamate (cyclohexyl sulfamic acid; cyclamic acid; XI) was approved as a food additive in the United States in 1949, and before their use was prohibited by the U.S. FDA in late 1969, the sodium and calcium salts and the acid form of cyclamic acid were widely employed as sweeteners. Cyclamates are about 30 times sweeter than sucrose, taste much like sucrose without significant interfering taste sensations, and are heat stable. Cyclamate sweetness has a slow onset, and persists for a period of time that is longer than that for sucrose.

Some early experimental evidence with rodents had suggested that cyclamate and its hydrolysis product, cyclohexylamine (Figure 11.15), caused bladder cancer [5,45]. However, extensive testing subsequently has not substantiated the early reports, and petitions have been filed in the United States for reinstatement of cyclamate as an approved sweetener [40]. Currently, cyclamate is permitted for use in low-calorie foods in some 40 countries and Canada. Still, for various reasons, even though extensive data support the conclusion that neither cyclamate nor cyclohexylamine are carcinogenic or genotoxic [4], the U.S. FDA has not reapproved cyclamates for use in foods.

The calcium and sodium salts and free acid form of saccharin (3-oxo-2,3-dihydro-1,2-benzisothiazole-1,1-dioxide; XII) are available as non-nutritive sweeteners. The commonly accepted



Sodium cyclamate

Cyclohexylamine

FIGURE 11.15 Formation of cyclohexylamine by the hydrolysis of cyclamate.

rule of thumb is that saccharin is about 300 times as sweet as sucrose in concentrations up to the equivalent of a 10% sucrose solution, but ranges from 200 to 700 times the sweetness of sucrose depending on the concentration and the food matrix [47]. Saccharin exhibits a bitter, metallic aftertaste, especially to some individuals, and this effect becomes more evident with increasing concentration.

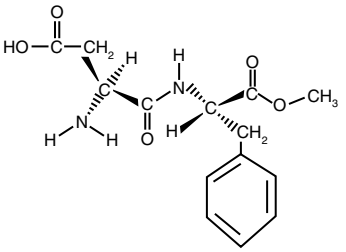
The safety of saccharin has been under investigation for over 50 years, and it has been found to cause a low incidence of carcinogenesis in laboratory animals. However, many scientists argue that the animal data are not relevant to humans. In humans, saccharin is rapidly absorbed and then is rapidly excreted in the urine. Although current regulations in the United States prohibit the use of food additives that cause cancer in any experimental animals, a ban on saccharin in the United States, proposed by the FDA in 1977, had been stayed by congressional legislation pending further research. In 2000, saccharin was delisted as a human carcinogen in the United States, which negated the health-warning statement required on packages of saccharin-containing foods. Saccharin is approved for use in more than 90 countries around the world.

Acesulfame K [6-methyl-1,2,3-oxathiazine-4(3H)-one-2,2-dioxide] (X) was discovered in Germany, and was first approved for use as a non-nutritive sweetener in the United States in 1988. The complex chemical name of this substance led to the creation of the trademarked common name, Acesulfame K, which is based on relationships to acetoacetic acid and sulfamic acid employed in its synthesis, and to its potassium salt nature.

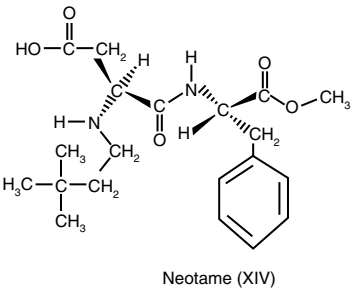
Acesulfame K is about 200 times as sweet as sucrose at a 3% concentration in solution, and it exhibits a sweetness quality between that of cyclamates and saccharin. Since Acesulfame K possesses some metallic and bitter taste notes at higher concentrations, it is especially useful when blended with other low-calorie sweeteners, such as aspartame. Acesulfame K is exceptionally stable at elevated temperatures encountered in baking, and it is also stable in acidic products, such as carbonated soft drinks. Acesulfame K is not metabolized in the body, thus providing no calories, and is excreted through the kidneys unchanged. Extensive testing has shown no toxic effects in animals, and exceptional stability in food applications.

11.8.2 Peptides: Aspartame, Neotame, and Alitame

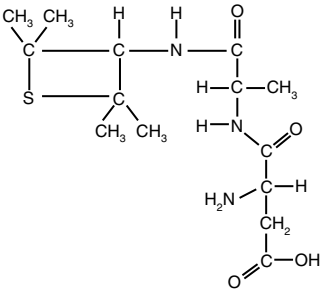
The peptide sweeteners have emerged in response to demands for sweetening ingredients to lower the caloric content of foods and beverages. Although the component amino acids of the peptide sweeteners become calorically available during digestive processes, their intense sweetness allows functionality to be achieved at very low levels that provide insignificant calories. Aspartame (XIII), Neotame (XIV), and Alitame (XV) comprise the group of peptide sweeteners that are permitted in foods in at least some countries.



Aspartame (XIII)
(L-Aspartyl-L-phenylalanine methyl ester)



Neotame (XIV)
(L-Phenylalanine, *N*-{*N*-(3,3-dimethylbutyl)-L-α-aspartyl}-,1-methyl ester)



Alitame (XV)
[L-α-Aspartyl-*N*-(2,2,4,4-tetramethyl-3-thietanyl)-D-alaninamide]

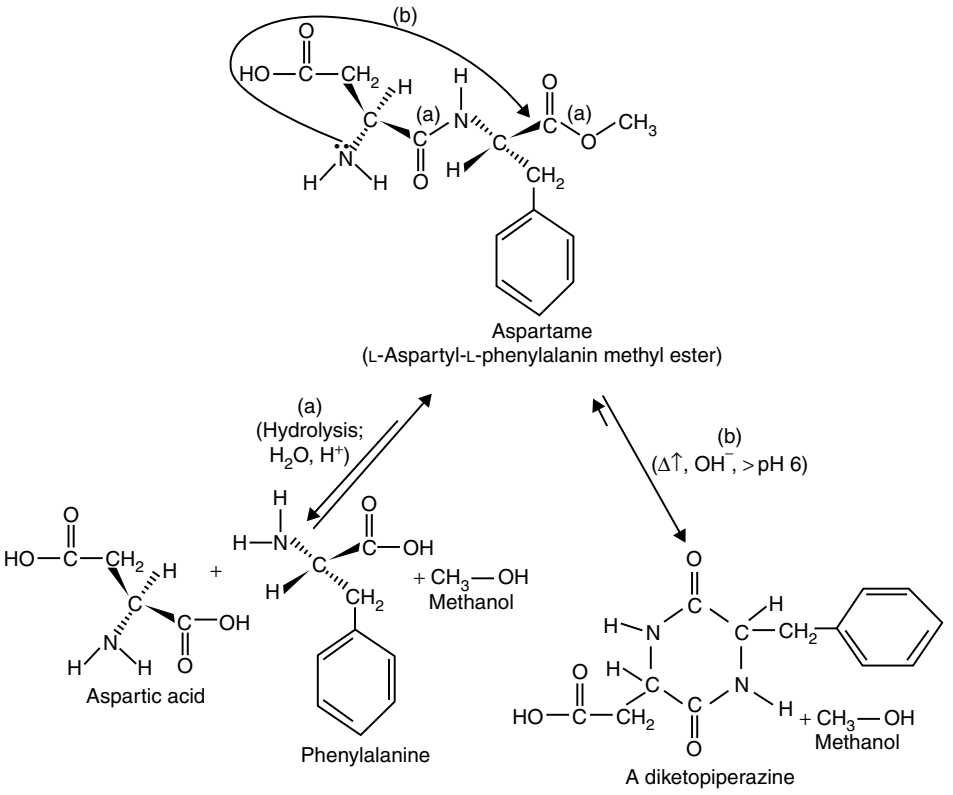


FIGURE 11.16 Reactions involved in the degradation of aspartame.

Aspartame (L-aspartyl-L-phenylalanine methyl ester; XIII) with a sweetening power of 180–200 times that of sucrose was first approved in the United States in 1981, and now is approved for use in over 75 countries where it is used in numerous products. It is noted for a clean, sweet taste although it lacks some of the sweetness qualities of sucrose. Two technical disadvantages of aspartame are its instability under acid conditions and its rapid degradation when exposed to elevated temperatures. Under acidic conditions, such as carbonated soft drinks, the rate of loss of sweetness is gradual, and depends on the temperature and pH. The peptide nature of aspartame makes it susceptible to hydrolysis (Figure 11.16), and this feature also permits other chemical interactions and microbial degradations. In addition to loss of sweetness resulting from hydrolysis of either the methyl ester on phenylalanine or the peptide bond between the two amino acids, aspartame readily undergoes an intramolecular condensation, especially at elevated temperatures, to yield the diketopiperazine (5-benzyl-3,6-dioxo-2-piperazine acetic acid) shown in Figure 11.16. This reaction is especially favored at neutral and alkaline pH values because nonprotonated amine groups on the molecule are more available for reaction under these conditions. Similarly, alkaline pH values promote carbonyl–amino reactions, and aspartame has been shown to react readily with glucose and vanillin under such conditions. With the glucose reaction, loss of aspartame's sweetness during storage is the principal concern while loss of vanilla flavor is the main concern in the latter case.

Even though aspartame is composed of naturally occurring amino acids and its daily intake is projected to be very small (0.8 g per person), concern has been expressed about its potential safety as a food additive. Aspartame-sweetened products must be labeled prominently about their phenylalanine content to allow avoidance of consumption by phenylketonuric individuals who lack 4-monooxygenase that is involved in the metabolism of phenylalanine. Concern also has been expressed by some about the potential long-term toxicological effects of the methanol that is released

via hydrolysis of the methyl ester. This health issue relates to the potential for metabolic conversion of methanol to formaldehyde. However, substantial methanol that is released from pectin polymers in plant-based foods (Chapter 3) also is consumed without apparent toxic effects. Similarly, consumption of aspartame by the normal population is not associated with adverse health effects. Although criticized by some, extensive testing has similarly shown that the diketopiperazine from aspartame does not pose risks to humans at concentrations potentially encountered in foods [29].

Neotame (L-phenylalanine, *N*-{*N*-(3,3-dimethylbutyl)-L-α-aspartyl}-,1-methyl ester; XIV] is structurally related to aspartame (XIII), and was approved for use in foods in the United States in 2002. Neotame was developed as an ingredient because it exhibits increased stability to conditions encountered in food preparation and its very high sweetening power (7,000–13,000 times that of sucrose) that permits usage without the necessity for cautionary labeling for phenylketonurics. The intense sweetness of neotame compared to that of aspartame is derived in a large part from the addition of the 3,3-dimethylbutyl-substituent to the amino group of the aspartic acid moiety of aspartic acid. This supplements the γ-grouping of aspartame with a strongly hydrophobic component that promotes intense sweetness (Chapter 10). Because usage of very low levels of neotame often exhibits beneficial effects on the flavors of some foods, it is also marketed as a flavor enhancer (Chapter 10).

Alitame [L-α-aspartyl-*N*-(2,2,4,4-tetramethyl-3-thietanyl)-D-alaninamide; XV] is an amino-acid-based sweetener that possesses a sweetening power of about 2,000 times that of sucrose, and it exhibits a clean sweet taste similar to sucrose. It is highly soluble in water, and has good thermal stability and shelf life, but prolonged storage in some acidic solutions may result in off-flavors. Generally, alitame has the potential for use in most foods where sweeteners are employed, including baked goods.

Alitame is synthesized from the amino acids, L-aspartic acid and D-alanine, and a novel amine. The alanine amide moiety of alitame apparently passes through the body with minimal metabolic changes. Extensive testing indicates that alitame is safe for human consumption, and a petition for its use in foods was filed in 1986 with the U.S. FDA. Although not yet approved for use in foods in the United States, alitame has been approved in Australia, New Zealand, China, and Mexico.

11.8.3 Chlorosaccharides: Sucralose

Chlorosaccharides are synthesized by combining selective chlorination of saccharide (sugar) molecules with other synthetic strategies, such as directed condensations, to yield molecules that may possess intense sweetness. The chlorosaccharide, sucralose (1,6-dichloro-1,6-dideoxy-β-fructofuranosyl-4-chloro-α-D-galactopyranoside), was approved in 1998 and 1999 for broad food uses in the United States, and currently has also been approved for use in more than 40 countries.

Sucralose is about 600 times sweeter than sucrose, and possesses a sweetness time–intensity profile similar to sucrose with no bitterness or other unpleasant aftertastes, it exhibits a high degree of crystallinity, high water solubility, and very good stability at high temperatures. It is also quite stable at the pH of carbonated soft drinks, and only limited hydrolysis to monosaccharide units occurs during usual handling and storage of these products.

The sucralose molecule is designed to resist digestive and metabolic attack by providing molecular features that are not easily recognized by constitutive hydrolytic enzymes. The molecular features contributing to this stability are shown in Figure 11.17 where they are compared with those of the naturally occurring counterpart saccharides, sucrose, and lactose (Chapter 3).

In addition to the replacement of three hydroxyl groups with chlorine atoms on the sucralose molecule, it also possesses a glycosidic β-linkage between its galactose and fructose moieties (Figure 11.17a). Comparison of counterpart features in sucrose (Figure 11.17b) and lactose (Figure 11.17c) reveal a mixing of the two basic structures in sucralose that preclude recognition by usual digestive and metabolic enzymes. However, some hydrolysis of the sucralose molecule has been reported to occur during digestion, mediated either by acid-catalyzed or microbial enzymic processes (Figure 11.18).

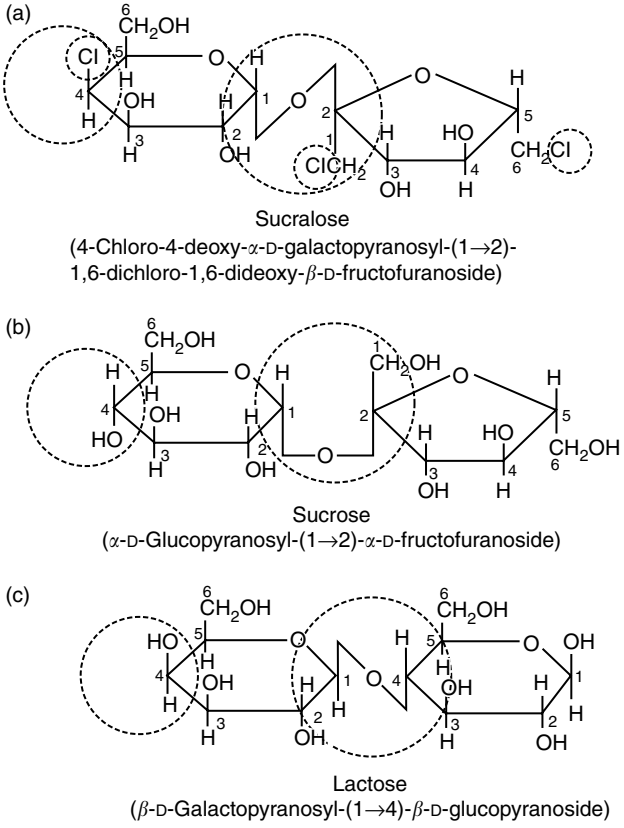


FIGURE 11.17 Comparison of structural features of (a) sucralose, (b) sucrose, and (c) lactose.

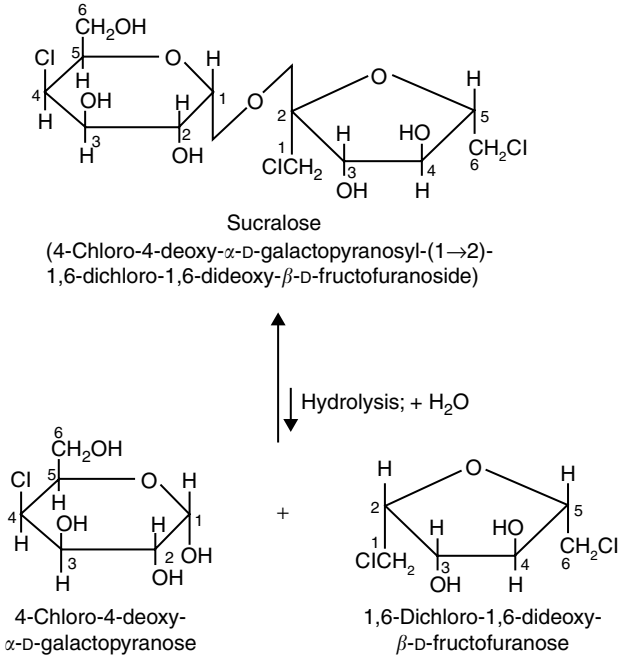


FIGURE 11.18 Reaction showing the hydrolysis products of sucralose.

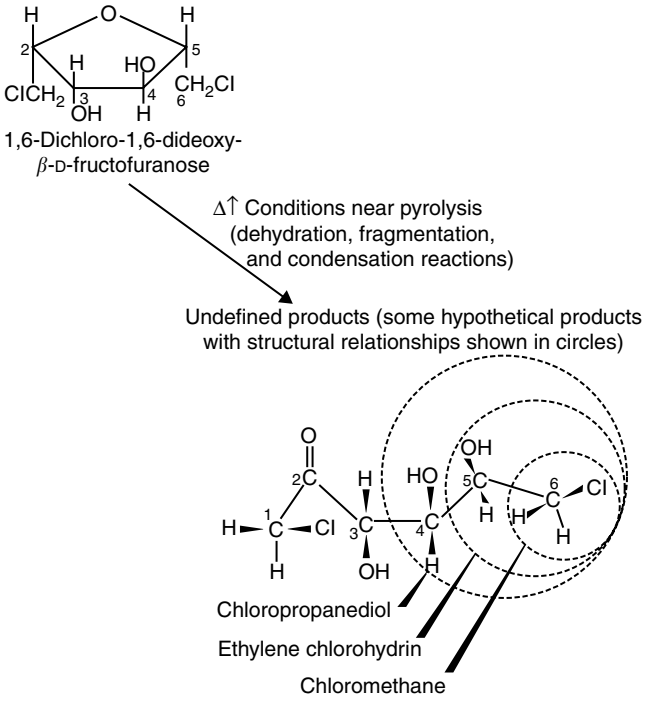


FIGURE 11.19 Structures of potentially harmful substances contained within the overall structure of 1,6-dichloro-1,6-dideoxy-β-D-fructofuranose.

Extensive studies have been conducted on the safety of sucralose, and these have generally demonstrated that the substance is safe at the expected usage levels. However, some have criticized the approval of sucralose as premature because it contains structural components of harmful substances that might be formed, especially upon exposure to thermal degradative conditions (Figure 11.19).

11.8.4 Other Intensely Sweet Non-Nutritive or Low-Calorie Sweeteners

The intensive search for alternative sweeteners over the past two decades has led to the discovery of a large number of new sweet compounds, and some of these are undergoing further development and safety studies to determine if they are suitable for future commercialization. These compounds join a substantial list of less well known, but emerging intensely sweet compounds, and some of the latter group are discussed here.

Glycyrrhizin (glycyrrhizic acid) is a triterpene saponin that is found in licorice root, and is 50–100 times sweeter than sucrose. Glycyrrhizin is a glycoside that on hydrolysis yields two moles of glucuronic acid and one mole of glycyrrhetinic acid, a triterpene related to aleanolic acid. Ammonium glycyrrhizin, the fully ammoniated salt of glycyrrhizic acid, is commercially available, and is approved for use only as a flavor and as a surfactant, but not as a sweetener. Glycyrrhizic acid is used primarily in tobacco products and to some extent in foods and beverages. Its licorice-like flavor influences its suitability for some applications.

A mixture of glycosides found in the leaves of the South American plant, *Stevia rebaudiana* Bertoni, is the source of stevioside and rebaudiosides. Pure stevioside is about 300 times as sweet as sucrose. Stevioside exhibits some bitterness and undesirable aftertastes at higher concentrations, and rebaudioside A exhibits the best taste profile of the mixture. However, extracts produced from

S. rebaudiana are used as commercial forms of this sweetener, and they are employed extensively in Japan. Extensive safety and toxicological testing have indicated that the extracts are safe for human consumption, but they are not approved in the United States.

Neohesperidin dihydrochalcone is a non-nutritive sweetener that is 1,500–2,000 times as sweet as sucrose, and it is derived from the bitter flavonones of citrus fruit. Neohesperidin dihydrochalcone exhibits a slow onset in sweetness and a lingering sweet aftertaste, but it decreases the perception of concurrent bitterness. This intensely sweet substance as well as other similar compounds are produced by hydrogenation of (l) naringin to yield naringin dihydrochalcone, (2) neohesperidin to yield neohesperidin dihydrochalcone, or (3) hesperidin to yield hesperidin dihydrochalcone 4′-*O*-glucoside [44]. Neohesperidin dihydrochalcone has been extensively tested for safety, and the studies have generally confirmed its safety. It is approved for use in Belgium and Argentina, but the U.S. FDA has requested additional toxicology testing.

Several sweet proteins are now known, and Thaumatin I and II obtained from the tropical African fruit katemfe (*Thaumatococcus daniellii*) have been well characterized. Thaumatin I and II are alkaline proteins, each with a molecular weight of about 20,000 [58], and on a mass basis they are about 1,600–2,000 times as sweet as sucrose. An extract of katemfe fruit is marketed under the tradename of Talin in the United Kingdom, and its use as a sweetener and flavor enhancer has been approved in Japan and Great Britain. It is also permitted as a flavor enhancer in chewing gum in the United States. Talin exhibits long-lasting sweetness with a slight licorice-like taste that limits its use along with its high costs.

Monellin is a sweet protein obtained from the serendipity berry, and it has a molecular weight of about 11,500. Monellin is about 3,000 times as sweet as sucrose on a mass basis, and the sweetness of natural monellin is destroyed by boiling. The potential use of sweet proteins is limited because the compounds are expensive, unstable to heat, and lose sweetness when held in solution below pH 2 at room temperature.

Brazzein is a sweet plant protein (54 amino acid residues) that was initially discovered in the fruits of the African vine, *Pentadiplandra brazzeana*. Field corn varieties have been genetically engineered to produce the sweet protein, and efforts are underway to commercially produce the sweetener for food uses by extracting it from the germ of corn seeds. It is reported to be quite stable and possess both sweetness and desirable mouthfeel characteristics.

Another basic protein, miraculin, has been isolated from miracle fruit (*Richadella dulcifica*), and this protein is tasteless. However, it has the peculiar property of changing sour taste into sweet taste, that is, it makes lemons taste sweet. Miraculin is a glycoprotein with a molecular weight of 42,000 [58], and similar to other protein sweeteners, miraculin is heat labile and inactivated at low pH values. The sweetness induced by 0.1 M citric acid after tasting 1 mM miraculin solution is equivalent to a 0.4 M sucrose solution; thus, the sweetness of a miraculin solution induced by 0.1 M citric acid solution has been calculated to be 400,000 times that of a sucrose solution. The taste effects of miraculin persist for over 24 h after placing it in the mouth, and this limits its potential use. In the 1970s, miraculin was introduced in the United States as a sweetening aid for diabetics, but it was banned by the U.S. FDA because of insufficient safety data.

11.9 POLYOLS: SWEETENERS, TEXTURIZERS, AND EMULSIFIERS

Simple polyols or polyhydric alcohols are structural analogs of carbohydrates that contain only hydroxyl groups as functional groups (Chapter 3). Thus, simple sugars and polyhydric alcohols (sugar alcohols) are structurally similar, except that sugars also contain aldo or keto groups (free or bound) that may adversely affect their chemical stability, especially at high temperatures.

Polyols are generally quite water soluble, hygroscopic materials that exhibit moderate viscosities at high concentrations in water, and the polyhydroxy structures of these compounds provide

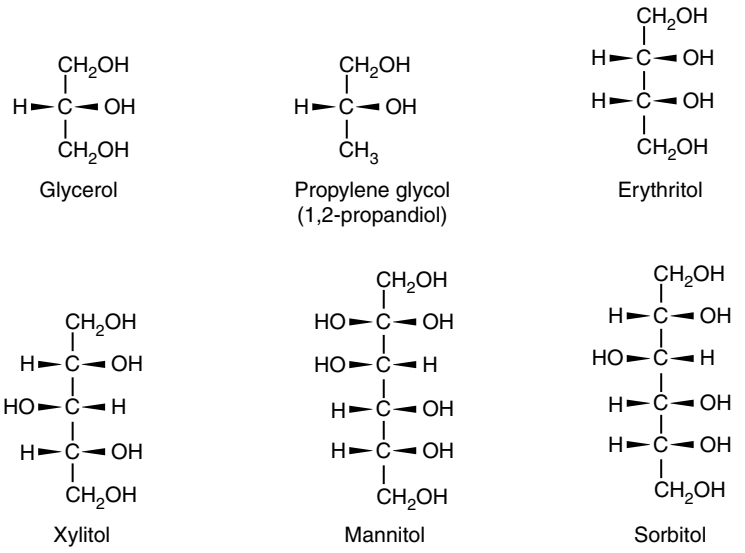


FIGURE 11.20 Comparative structures of simple polyhydric alcohols used as food ingredients.

water-binding properties that have been exploited in foods. Specific functions of polyhydric alcohols include control of viscosity and texture, addition of bulk, retention of moisture, reduction of water activity, control of crystallization, improvement or retention of softness, improvement of rehydration properties of dehydrated foods, and use as a solvent for flavor compounds [24]. Many applications of polyhydric alcohols in foods rely on concurrent contributions of functional properties from sugars, proteins, starches, and gums.

Some simple polyhydric alcohols occur naturally, but because of their limited concentrations, they often do not contribute functional roles in food. For example, free glycerol exists in wine and beer as a result of fermentation, and sorbitol occurs in fruits such as pears, apples, and prunes. While the number of available simple polyhydric alcohols is substantial, relatively few have been important in food applications (Figure 11.20).

Simple polyols (sugar alcohols) generally are sweet, but less so than sucrose (Table 11.4). Very short-chain members, such as glycerol, are slightly bitter at high concentrations. When used in the dry form, simple polyols often contribute a pleasant cooling sensation because of their negative heat of solution. Notably, the usage of some polyhydric alcohols is growing because of demands for their reduced-calorie sweetener properties. Historically, the energy value of simple polyols derived from sugars, like sugars, has been considered to be 16.7 kJ (4 kcal) g^{-1} (joules = calories $\times$ 4.1816) for labeling puposes in the United States. However, this view has changed very recently following a 1990 European Union lead of assigning an energy value of 10 kJ (2.4 kcal) g^{-1} to polyols as a group. The U.S. FDA has accepted caloric contents ranging from 6.7 to 12.5 kJ (1.6–3.0 kcal) g^{-1} for the various commercially available polyols (Table 11.4). This has markedly changed the positioning of polyols as food ingredients, and it can be anticipated that their presence in low-calorie, reduced-fat, and sugar-free foods will continue to increase in the future. Although there is some lingering controversy relating to the influence of polyols on diabetics, the currently accepted general philosophy is that they are suitable in diets of these individuals.

Xylitol, sorbitol, mannitol, maltitol, and lactitol are manufactured by hydrogenation of xylose, glucose (Figure 11.21), mannose, maltose, and lactose, respectively. Hydrogenated starch hydrolysates also are employed as food ingredients, especially in confections, and these contain sorbitol from glucose, maltitol from maltose, and various polymeric polyols (hydrogenated maltodextrins) from oligosaccharides. Isomalt is derived from sucrose using a multiple-step process (Figure 11.22). The 1- to 2-glycosidic linkage of sucrose is first enzymically isomerized to 1–6 linkages between the

TABLE 11.4
Relative Sweetness and Energy Values of Some Relatively Simple Polyols and Sugars

Substance	Relative Sweetness[a] (sucrose = 1, wt. basis)	Energy Value[b] (kJ g^{-1})
Simple polyols		
Erythritol	0.7	0.84
Mannitol	0.6	6.69
Lactitol	0.3	8.36
Isomalt	0.4–0.6	8.36
Xylitol	1.0	10.03
Sorbitol	0.5	10.87
Maltitol	0.8	12.54
Hydrogenated corn syrup	0.3–0.75	12.54
Sugars		
Xylose	0.7	16.72
Glucose	0.5–0.8	16.72
Fructose	1.2–1.5	16.72
Galactose	0.6	16.72
Mannose	0.4	16.72
Lactose	0.2	16.72
Maltose	0.5	16.72
Sucrose	1.0	16.72

[a] Commonly cited relative sweetness values are listed; however, the concentration and the food or beverage matrix may greatly influence actual relative sweetness values for sweeteners.
[b] Energy values accepted by the U.S. FDA; 1 kcal = 4.1816 kJ.

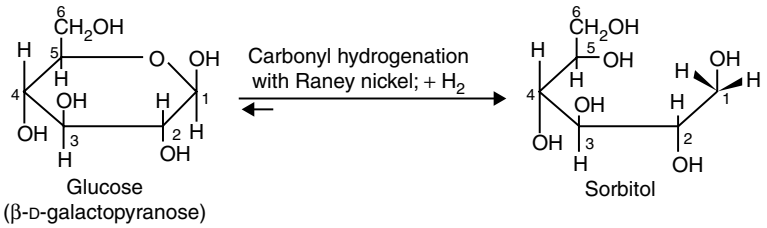


FIGURE 11.21 Reaction showing the hydrogenation of glucose for the manufacture of sorbitol.

glucose and fructose moieties, respectively. Subsequent hydrogenation of this intermediate results in an equimolar mixture of two disaccharide polyols, gluco-mannitol, and gluco-sorbitol.

Simple polyols may also provide the starting materials for the manufacture of other food ingredients, such as emulsifiers (Chapter 13). An example of this is the use of sorbitol as a reactant in the manufacture of spans and tweens (Figure 11.23). Sorbitol is first converted to a sorbitan, and then is esterified to a fatty acid (stearic acid in this case) to provide an amphiphilic property to the resulting sorbitan monostearate (span) molecule. The remaining hydroxyl groups on sorbitan monostearate can then provide reaction sites for adding repeating ether linked moieties from ethylene oxide to yield polysorbate (tween) emulsifiers.

Large molecular weight polymeric forms of polyhydric alcohols have also been developed for food applications. Whereas ethylene glycol ($CH_2OH{-}CH_2OH$) is toxic, polyethylene glycol 6000 is allowed in some food coating and plasticizing applications. Polyglycerol [$CH_2OH{-}CHOH{-}CH_2{-}(O{-}CH_2CHOH{-}CH_2)_n{-}O{-}CH_2{-}CHOH{-}CH_2OH$], formed from

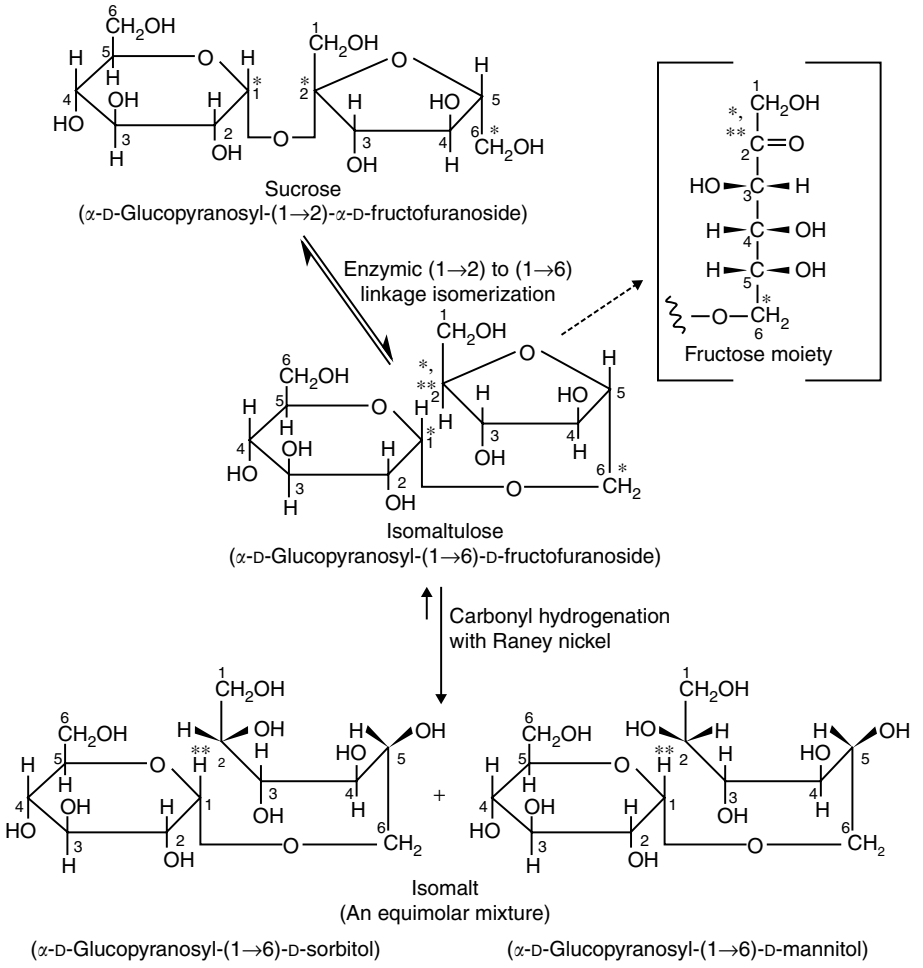


FIGURE 11.22 Reactions utilized in the manufacture of isomalt.

glycerol through an alkaline catalyzed polymerization, also exhibits useful properties. It can be further modified by esterification with fatty acids to yield materials with lipid-like characteristics. These polyglycerol materials have been approved for food use because the hydrolysis products, glycerol and fatty acids, are metabolized normally.

Intermediate moisture (IM) foods deserve some discussion since polyhydric alcohols can make an important contribution to the stability of these products. IM foods contain substantial moisture (15–30%), yet are stable to microbiological deterioration without refrigeration. Several familiar foods, including dried fruits, jams, jellies, marshmallows, fruit cake, and jerky, owe their stability to IM characteristics [35]. Some of these items may be rehydrated before consumption, but all possess a plastic texture and can be consumed directly. Although moist shelf-stable pet foods have found ready acceptance, new forms of IM foods for human consumption have not as yet become popular. Nevertheless, meat, vegetable, fruit, and combination prepared dishes are under development and may eventually become important forms of preserved foods.

Most IM foods possess water activities of 0.70–0.85 and those containing humectants contain moisture contents of about 20 g of water per 100 g of solids (82% H_2O by weight). If IM foods with a water activity of about 0.85 are prepared by desorption, they are still susceptible to attack by molds and yeasts. To overcome this problem, the ingredients can be heated during preparation and an antimycotic agent, such as sorbic acid, can be added.

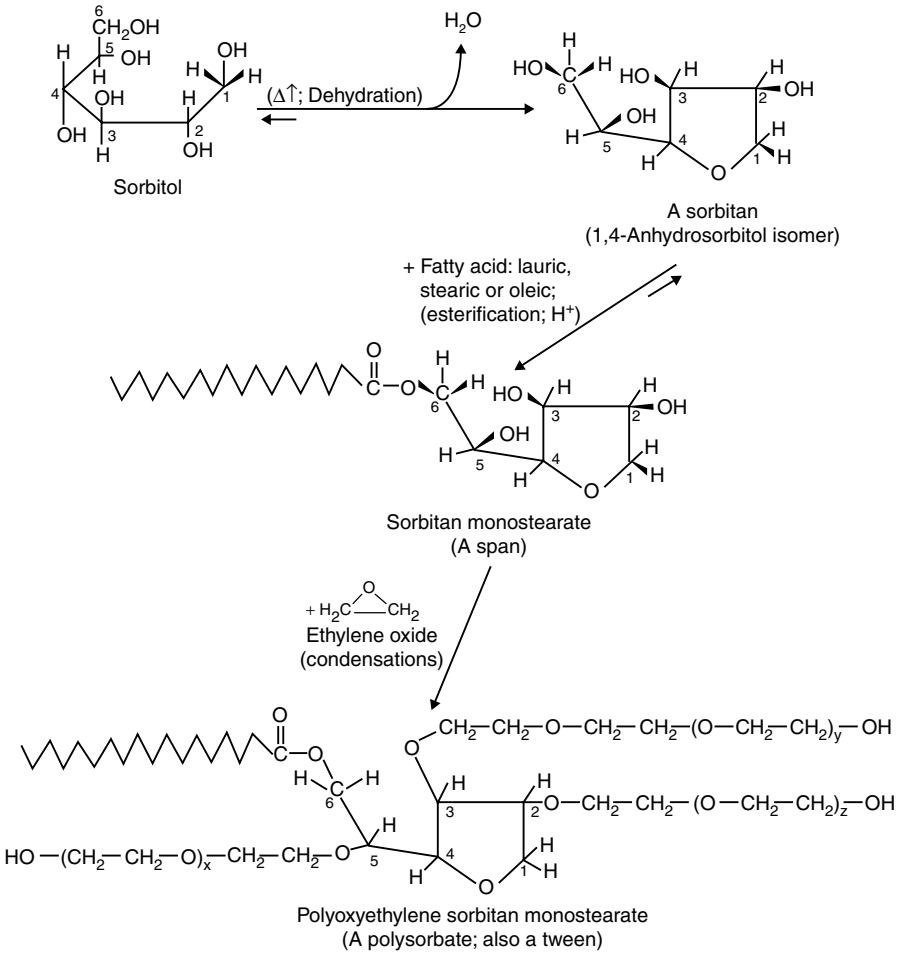


FIGURE 11.23 Reactions showing the use of sorbitol in the manufacture of span and tween (polysorbate) emulsifiers.

To obtain the desired water activity, it is usually necessary to add a humectant that binds water and maintains a soft palatable texture. Relatively few substances, mainly glycerol, sucrose, glucose, propylene glycol, and sodium chloride, are sufficiently effective in lowering the water activity while being tolerable organoleptically to be of value in preparing IM foods. On the other hand, the formulation technology for most contemporary sugar-free candies relies on fundamental IM principles.

11.10 STABILIZERS AND THICKENERS

Many hydrocolloid materials are widely used for their unique textural, structural, and functional characteristics in foods where they provide stabilization for emulsions, suspensions, and foams, and for general thickening properties. Most of these materials, sometimes classed as gums, are derived from natural sources although some are chemically modified to achieve desired characteristics. Many stabilizers and thickeners are polysaccharides, such as gum arabic, guar gum, carboxymethylcellulose, carrageenan, agar, starch, and pectin. The chemical properties of these and related carbohydrates are discussed in Chapter 3. Gelatin, a protein derived from collagen, is one of the few noncarbohydrate stabilizers used extensively and it is discussed in Chapter 5. All effective stabilizers and thickeners are hydrophilic and are dispersed in solution as colloids, which leads to the designation hydrocolloid. General properties of useful hydrocolloids include significant solubility in

water, a capability to increase viscosity, and in some cases an ability to form gels (Chapter 3). Some specific functions of hydrocolloids include improvement of texture, inhibition of crystallization (sugar and ice), stabilization of emulsions and foams, improvement (reduced stickiness) of icings on baked goods, and encapsulation of flavors [31]. Hydrocolloids are generally used at concentrations of about 2% or less because many exhibit limited dispersibility, and the desired functionality is provided at these levels. The efficacy of hydrocolloids in many applications is directly dependent on their ability to increase viscosity. For example, this is the mechanism by which hydrocolloids stabilize oil-in-water emulsions. They cannot function as true emulsifiers since they lack the necessary combination of strong hydrophilic and lipophilic properties within individual molecules.

11.11 FAT REPLACERS

Although fat is an essential dietary component, too much fat in the diet has been linked with a higher risk of coronary heart disease and certain types of cancer. Consumers are being advised to eat lean meats, especially fish and skinless poultry, low-fat dairy products, and to restrict their consumption of fried foods, high-fat baked goods, and sauces and dressings. However, consumers want substantially reduced-calorie foods that possess the sensory properties of traditional high-fat foods.

While the increasing availability of complex prepared foods has contributed to the overabundance of fat in the diets of developed countries, it also has provided an opportunity to develop the complex technologies required for the manufacture and mass-marketing of reduced-fat foods that simulate full-fat counterparts. Over the past two decades, a great deal of progress has been made in the adaptation and development of ingredients for use in reduced-fat foods. The types of ingredients suggested for various reduced-fat food applications vary widely, and are derived from several chemical groups, including carbohydrates, proteins, lipids, and purely synthetic compounds.

When fat is either partially or completely omitted from foods, the properties of the foods are altered, and it is necessary to replace it by some other ingredient or component. Hence, the term, "fat replacers," has been spawned to broadly indicate the ingredients that are functionally used in this capacity. When the substances provide identical physical and sensory properties to fats, but without calories, they are designated, "fat substitutes." These ingredients convey both fat-like sensory properties in foods, and perform physically in various applications, such as in frying foods.

Other ingredients that do not possess full functional equivalency to fats, are termed, "fat mimetics," because they can be made to mimic the effects of fat in certain applications. An example of this is the simulation of the pseudo-moistness provided by fat to certain high-fat bakery products. Certain substances, such as specially modified starches, can be used to provide the desired simulated fat properties by contributing to sensory properties arising from bulking and moisture retention.

11.11.1 Carbohydrate Fat Mimetics

Modestly processed starches, gums, hemicelluloses, and cellulose are used in many forms for providing partial fat functionality in reduced fat foods, and the chemistry of these substances is discussed in Chapter 3, and in Sections 11.9 and 11.10. Additional information about their applications in reduced fat foods can also be found in reviews [2,37]. Generally, some carbohydrate fat mimetics provide essentially no calories (e.g., gums, celluloses) while others provide up to 16.7 kJ (4 kcal) g^{-1} (e.g., modified starches) rather than the 37.6 kJ (9 kcal) g^{-1} of traditional fats. These substances mimic the smoothness or creaminess of fats in foods primarily by moisture retention and bulkiness of their solids that assist in providing fat-like sensations, such as moistness in baked goods and the textural bite of ice cream.

11.11.2 Protein Fat Mimetics

Several attempts have been made to exploit selected proteins (Chapter 5) as fat mimetics [39], and they generally have GRAS (generally recognized as safe; U.S.) approval. However, the functionality

of these proteins as fat mimetics is limited because they do not perform like fats at highly elevated temperatures, such as is required in frying applications. Nevertheless, some of these protein (16.7 kJ [4 kcal] g^{-1}) ingredients may be useful for replacing fat in foods, especially in oil-in-water emulsions. For these applications, they can be variously prepared into microparticulates ($<3\ \mu m$ diameter) where they simulate the physical nature of fats through a means that has been described as being similar to flexible ball bearings. Proteins in solution also provide thickening, lubricity, and mouthcoating effects. Gelatin is quite functional in reduced-fat, solid products, such as margarine, where it provides thermally reversible gelation during manufacture, and subsequently it provides thickness to the margarine mass.

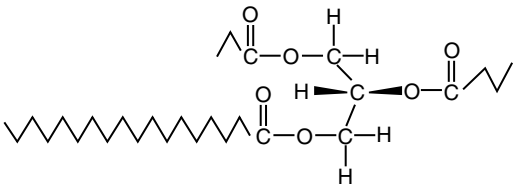
The manufacture of protein-based fat mimetics involves several strategies that each utilize soluble proteins as the starting materials. Particulate proteins are obtained from soluble proteins by inducing one of the following events: (1) hydrophobic interactions, (2) isoelectric precipitation, (3) heat denaturation and/or coagulation, (4) protein–protein complex formation, or (5) protein–polysaccharide complex formation [39]. These processes are often accompanied by physical shearing action that assures the formation of microparticles.

11.11.3 Reduced-Calorie Synthetic Triacylglycerol Fat Substitutes

Attempts have been made to take advantage of certain triglycerides (triacylglycerols; Chapter 4), which because of unique structural features do not yield full caloric value when consumed by humans and other monogastrics. These triglycerides are variously synthesized utilizing hydrogenation and directed esterifications or interesterifications. One member of this group of lipids is the medium-chain triglycerides (MCTs), which have long been used in the treatment of certain lipid metabolism disorders. MCTs are composed of saturated fatty acids with chain lengths of C_6—C_{12}, and they provide about 34.7 (8.3 kcal) g^{-1} compared with regular triglycerides that contain 37.6 kJ (9 kcal) g^{-1} [34].

The incorporation of saturated short-chain fatty acids (C_2—C_5) along with a long-chain saturated fatty acid (C_{14}—C_{24}) in a triglyceride molecule is another strategy, and this greatly reduces the caloric value. The caloric reduction results in part because short-chain fatty acids provide fewer calories per unit weight than long-chain fatty acids. In addition, the position of the long-chain fatty acid on the glycerol molecule greatly influences the absorption of the long-chain fatty acid. In some positional combinations of short- and long-chain saturated fatty acids, the absorption of the long-chain fatty acid may be reduced by over half (see Chapter 4).

A family of triglycerides based on the above principles, tradenamed Salatrim (**S**hort **a**nd **l**ong **a**cyl**tri**glyceride **m**olecule), has been developed [52]. Salatrim (XVI) is a mixture of triglycerides composed of mainly stearic acid (C18) as the long-chain fatty acid obtained from hydrogenated vegetable fats and various proportions of acetic, propionic, and butyric acids (C_2, C_3, and C_4, respectively) as the short-chain fatty acids. Humans realize between 19.6 and 21.3 kJ (4.7 and 5.1 kcal) g^{-1} for various Salatrim products, and the fatty acid composition can be controlled to provide the desired physical properties, such as melting points.



Salatrim isomer (XVI)
(1-Propionyl-2-butyrl-3-stearoyl-*sn*-glycerol)

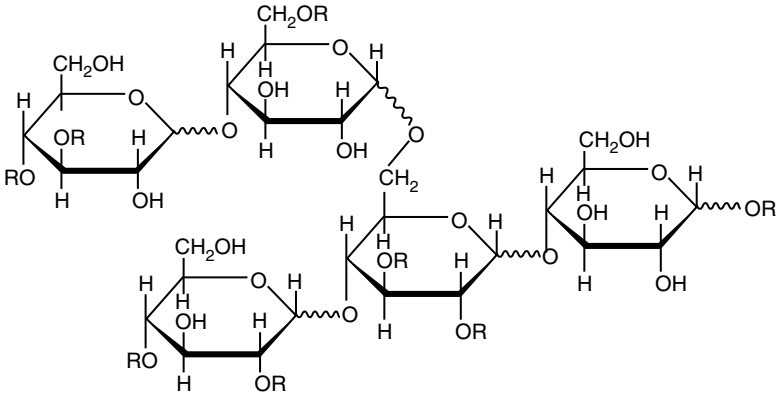
Caprenin is the tradename of a similarly synthesized, reduced-calorie triglyceride (about 20.9 kJ [5 kcal] g^{-1}) product that contains the medium-chain fatty acids, caprylic (C_6) and capric (C_{10}) acids, along with the long-chain fatty acid, behenic acid (C_{22}). Caprenin has been used in candy bars. Caprylic and capric acids are obtained from coconut and palm oils, and behenic acid can be obtained from hydrogenated marine oils, hydrogenated rapeseed oil, and peanut oil. Peanut oil contains about 3% behenic acid, whereas rapeseed oil contains about 35% erucic acid ($C_{22:1}$), which is converted to behenic acid by hydrogenation. Marine oils often contain over 10% of docosahexaenoic acid (DHA), which is also converted to behenic acid by hydrogenation.

11.11.4 Synthetic Fat Replacers

A great number of synthetic compounds have been found to provide either fat mimetic or fat substitute properties [1,2]. Many of them contain triacylglycerol-like structural and functional groups, such as the trialkoxycarballates, which in effect have the ester groups reversed compared to conventional fats (i.e., a tricarboxylic acid is esterified to saturated alcohols rather than glycerol being esterified to fatty acids). Owing to their synthetic nature, these compounds are resistant to enzymic hydrolysis, and are largely undigested in the gut. U.S. FDA approval for many of these substances is proving to be difficult to obtain, and it remains to be seen whether some of these compounds will find an ultimate role in the food supply.

11.11.4.1 Polydextrose

Although principally used as a reduced-calorie, carbohydrate bulking ingredient, polydextrose (XVII) behaves as a fat mimetic in some applications. Since polydextrose yields only 4.18 kJ (1 kcal) g^{-1}, it is especially attractive as a dual purpose ingredient that reduces calories from carbohydrates as well as fats. Contemporary polydextrose (tradename Litesse) is manufactured by randomly polymerizing glucose (minimum 90%), sorbitol (maximum 2%), and citric acid, and it contains minor amounts of glucose monomer and 1,6-anhydroglucose [2]. To maintain suitable water solubility, the molecular weight of polydextrose polymers are controlled below 22,000.

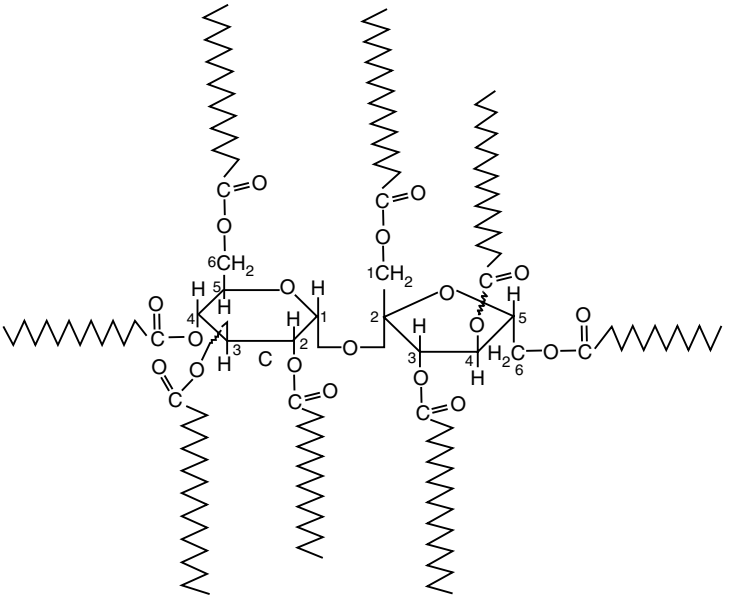


Polydextrose (XVII)

11.11.4.2 Sucrose Polyesters

Sucrose polyesters (XVIII) comprise a family of substances [1] that are formed through esterification of two or more of the eight available hydroxyl groups on the sucrose molecule. Some sucrose polyesters are found in nature, such as in the waxy coatings on leaves of some plants. Sucrose polyesters

are readily commercially manufactured by esterification of sucrose with fatty acids obtained from natural sources, and resulting polarity and melting point properties depend both on selection of fatty acids and degree of esterification. Sucrose polyesters exhibiting lower degrees of esterification possess amphiphilic properties that permit their use in emulsifier applications. Complete esterification of sucrose molecules, especially with longer-chain fatty acids, yields lipophilic, nondigestible, and nonabsorbable sucrose octaesters that possess physical and chemical properties of conventional fats.



A sucrose polyester isomer (XVII)

Although not used widely, sucrose polyester emulsifiers were approved for use in foods in the United States in 1983 with very little debate about health issues. This resulted because only small amounts of sucrose polyesters are employed in emulsifier applications, and they are readily digested because of their low degree of esterification. On the other hand, sucrose polyester fat replacers (octaesters; tradenames olestra and Olean) were deemed safe and approved for limited use as an industrial frying medium for snackfoods (e.g., potato and corn chips) by the U.S. FDA only in 1996 after over two decades of health and safety studies. The main health issues that caused the limited approval of sucrose polyester fat substitutes were much debated concerns about interferences with absorption of fat soluble vitamins and micronutrients as well as diarrhea and other disturbances caused by passage of excessive amounts of fat-like sucrose polyesters through the digestive tract.

11.12 MASTICATORY SUBSTANCES

Masticatory substances are employed to provide the long-lasting, pliable properties of chewing gum. These substances are either natural products or the result of organic synthesis, and both kinds are quite resistant to degradation. Synthetic masticatory substances are prepared by the Fischer–Tropsch process involving carbon monoxide, hydrogen, and a catalyst, and after further processing to remove low molecular weight compounds, the product is hydrogenated to yield synthetic paraffin [10]. Chemically modified masticatory substances are prepared by partially hydrogenating wood rosin, which is largely composed of diterpenes, and then esterifying the products with pentaerythritol or glycerol. Other polymers similar to synthetic rubbers have also been prepared for use as masticatory substances and these substances are prepared from ethylene, butadiene, or vinyl monomers.

Much of the masticatory base employed in chewing gum is derived directly from plant gums. These gums are purified by extensive treatments involving heating, centrifuging, and filtering. Chicle from plants in the *Sapotaceae* (Sapodilla) family, gums from *Gutta Katiau* from *Palaquium* sp., and latex solids (natural rubber) from *Henea brasiliensis* are the widely used, naturally derived, masticatory substances.

11.13 FIRMING TEXTURIZERS

Thermal processing or freezing of plant tissues usually causes softening because the cellular structure is modified. Stability and integrity of these tissues are dependent on maintenance of intact cells and firm molecular bonding between constituents of cell walls. The pectic substances (Chapters 3 and 17) are extensively involved in structure stabilization through cross-linking of their free carboxyl groups via polyvalent cations. Although considerable amounts of polyvalent cations are naturally present, calcium salts (0.1–0.25% as calcium) are frequently added. This increases firmness since the enhanced cross-linking results in increased amounts of relatively insoluble calcium pectinate and pectate. These stabilized structures support the tissue mass, and integrity is maintained even through heat processing. Fruits, including tomatoes, berries, and apple slices, are commonly firmed by adding one or more calcium salts before canning or freezing. The most commonly used salts include calcium chloride, calcium citrate, calcium sulfate, calcium lactate, and monocalcium phosphate. Most calcium salts are sparingly soluble, and some contribute a bitter flavor at higher concentrations.

Acidic alum salts, sodium aluminum sulfate [$NaAl(SO_4)_2 \cdot 12H_2O$], potassium aluminum sulfate, ammonium aluminum sulfate, and aluminum sulfate [$Al_2(SO_4)_3 \cdot 18H_2O$], are added to fermented, salt-brined pickles to make cucumber products that are crisper and firmer than those prepared without these salts. The trivalent aluminum ion is believed to be involved in the crisping process through the formation of complexes with pectin substances. However, some investigations have demonstrated that aluminum sulfate has a softening effect on fresh-pack or pasteurized pickles, and should not be included in these products [14]. The reasons for the softening are not understood, but the presence of aluminum sulfate counteracts the firming effects normally provided by adjusting the pH to near 3.8 with acetic or lactic acids.

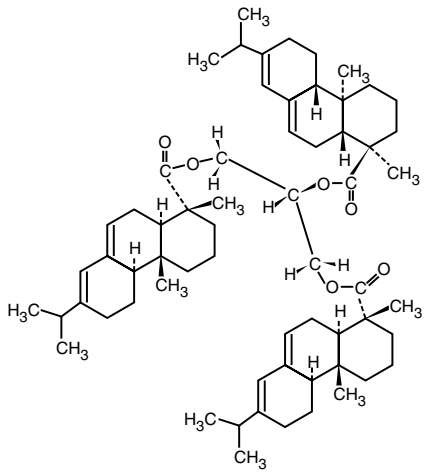
The firmness and texture of some vegetables and fruits can be manipulated during processing without the use of direct additives. For example, an enzyme, pectin methylesterase, is activated during low temperature blanching (70–82°C for 3–15 min) rather than inactivated as is the case during usual blanching (88–100°C for 3 min). The degree of firmness produced following low temperature blanching can be controlled by the holding time before retorting [57]. Pectin methylesterase hydrolyzes esterified methanol (sometimes referred to as methoxyl groups) from carboxyl groups on pectin to yield pectinic and pectic acids. Pectin, having relatively few free carboxyl groups, is not strongly bound, and because it is water soluble, it is free to migrate from the cell wall. On the other hand, pectinic acid and pectic acid possess large numbers of free carboxyl groups and they are relatively insoluble, especially in the presence of endogenous or added calcium ions. As a result, they remain in the cell wall during processing and produce firm textures. Firming effects through activation of pectin methylesterase have been obtained for snap beans, potatoes, cauliflower, and sour cherries as well as for a number of other fruits and vegetables. Addition of calcium ions in conjunction with enzyme activation leads to additional firming effects.

11.14 APPEARANCE CONTROL AND CLARIFYING AGENTS

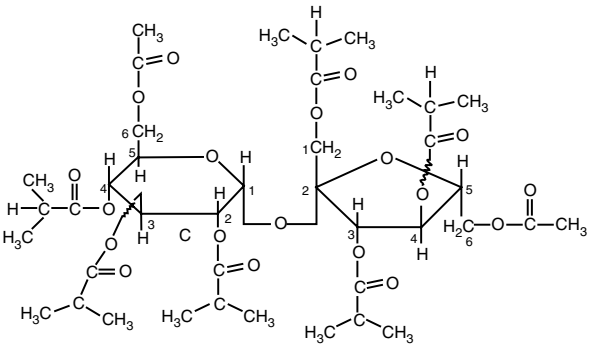
The appearance of beverage foods is critical to consumer acceptance, and maintenance of dispersions of colloidal particles or other entities is a key consideration for these products. In some instances, the physical appearance may be suitably maintained by slowing the settling of solids through ionic

associations and viscosity enhancement. Such is the case when carageenan (Chapter 3) is added to milk in the manufacture of chocolate milk that has been flavored with cocoa solids. In other cases, however, it is not practical or adequate to simply enhance the viscosity to stabilize the appearance of a fluid food or beverage. In such cases, alteration of the density of a dispersed phase may provide a convenient method to stabilize the appearance of the product.

Maintenance of the cloud provided by dispersed flavoring oils in soft drinks, particularly those composed of citrus oils (terpenes; Chapter 10), is achieved by increasing the density of the citrus oil phase (sp. gr. 0.85–0.90 g/cm^3) to near that of supporting sugar–water bulk phase (sp. gr. 1.04–1.05 g/cm^3). Historically, this has been achieved by dissolving a small amount of brominated vegetable oil (sp. gr. 1.23–1.33 g/cm^3) into the citrus oil flavoring employed. Brominated vegetable oils are prepared by reacting unsaturated vegetable oils with bromine (Figure 11.24). However, because of toxicological implications, alternative weighting agents are often substituted for brominated vegetable oils in citrus flavoring oil applications. Alternatives include the damar gums (sp. gr. about 1.05 g/cm^3) that are natural exudates obtained from shrubs of the *Caesalpinaceae* and *Dipterocarpacea* families, and ester gums, such as glyceryl triabietate (sp. gr. about 1.05 g/cm^3; XIX), that are manufactured from wood rosin. Sucrose diacetate hexaisobutyrate (sp. gr. 1.10–1.14 g/cm^3; XX) that is a synthesized sucrose polyester is also widely used. For comparison, when incorporated into soybean oil and dispersed in a standardized oil-in-water emulsion, iso-density (with water) concentrations (weight %) of weighting agents in soybean oil have been found to be 25, 45, 55, and 55 for brominated vegetable oil, sucrose diacetate hexaisobutryate, damar gum, and ester gum, respectively [7].



Glyceryl triabietate (XIX)



Sucrose diacetate hexaisobutyrate (XX)

In beer, wine, and many fruit juices the formation of hazes or sediments and oxidative deterioration have been long-standing problems. Natural phenolic substances are involved in these phenomena. The chemistry of this important group, including anthocyanins, flavonoids, proanthocyanidins, and tannins, is discussed in Chapter 9. Proteins and pectic substances participate with polyphenols in the formation of haze-forming colloids. Specific enzymes have been utilized to partially hydrolyze high molecular weight proteins (Chapter 6), and thereby reduce the tendency toward haze formation. However, in some instances excess enzymic activity can adversely affect other desirable properties, such as foam formation in beer.

An important means of manipulating polyphenolic composition to control both its desirable and undesirable effects is to use various clarifying ("fining") agents and adsorbants. Preformed haze can be at least partially removed by filter aids, such as diatomaceous earth. Many of the clarifying agents

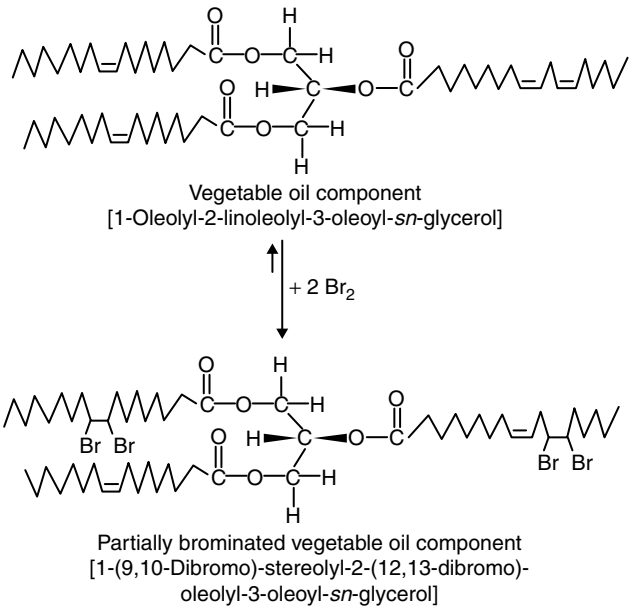


FIGURE 11.24 Reactions employed in the manufacture of brominated vegetable oils.

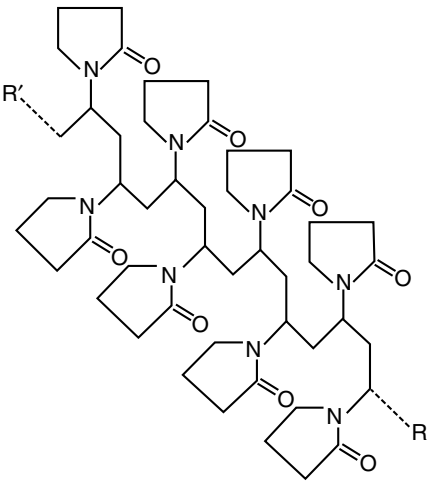
that have been used are nonselective and they affect the polyphenolic content more or less incidentally. Adsorption is usually maximal when solubility of the adsorbate is minimal, and suspended or nearly insoluble materials such as a tannin–protein complexes tend to collect at any interface. As the activity of the adsorbent increases, the less soluble substances still tend to be adsorbed preferentially, but more soluble compounds are also adsorbed.

Bentonite, a montmorillonite clay, is representative of many similar and moderately effective minerals that have been employed as clarifying agents. Montmorillonite is a complex hydrated aluminum silicate with exchangeable cations, frequently sodium ions. In aqueous suspension, bentonite behaves as small platelets of insoluble silicate. The bentonite platelets have a negative charge and a very large surface area of about 750 $m^2\ g^{-1}$. Bentonite is a rather selective adsorbent for protein, and evidently this adsorption results from an attraction between the positive charges of the protein and the negative charges of the silicate. A particle of bentonite covered with adsorbed protein will adsorb some phenolic tannins on or along with the protein [51]. Bentonite is used as a clarifying or fining agent for wines to preclude protein precipitation. Doses of the order of a few pounds per thousand gallons usually reduce the protein content of wine from 50 to l00 mg/L to a stable level of less than 10 mg/L. Bentonite rapidly forms a heavy compact sediment and is often employed in conjunction with final filtration to remove precipitated colloids.

The important clarifying agents that have a selective affinity for tannins, proanthocyanidins, and other polyphenols include proteins and certain synthetic resins, such as the polyamides and polyvinylpyrrolidone (PVP). Gelatin and isinglass (obtained from the swim-bladder of fish) are the proteins most commonly used to clarify beverages. It appears that the most important type of linkage between tannins and proteins, although probably not the only type, involves hydrogen bonding between phenolic hydroxyl groups and amide bonds in proteins. The addition of a small amount of gelatin (40–170 g per 380 L) to apple juice causes aggregation and precipitation of a gelatin–tannin complex that on settling enmeshes and removes other suspended solids. The exact amount of gelatin for each use must be determined at the time of processing. Juices containing low levels of polyphenolics are supplemented with added tannin or tannic acid (0.005–0.01%) to facilitate flocculation of the gelatin.

At low concentrations, gelatin and other soluble clarifying agents can act as protective colloids; at higher concentrations, they can cause precipitation and at still higher concentrations they can again fail to cause precipitation. Hydrogen bonding between the colloidal clarifying agents and water accounts for their solubilities. Molecules of the clarifying agent and polyphenol can combine in different proportions to either neutralize or enhance the hydration and solubility of a given colloidal particle. The most nearly complete disruption of H-bonding between water and either the protein or the polyphenol gives the most complete precipitation. This would be expected to occur when the amount of dissolved clarifying agent roughly equals the weight of the tannin being removed.

The synthetic resins (polyamides and PVP) have been used to prevent browning in white wines [6] and to remove haze for beers [12]. These polymers are available in both soluble and insoluble forms, but requirements for little or no residual polymer in beverages has stimulated use of the high-molecular-weight cross-linked forms that are insoluble. The synthetic resins have been particularly useful in the brewing industry where reversible refrigeration-induced haze (chill-haze) and permanent haze (that which is associated with the development of oxidized flavors) are serious problems. These hazes are caused by formation of complexes between native proteins and proanthocyanidins from malted barley. Excessive removal of proteins leads to defective foam character, but the selective removal of polyphenols extends the stability of beer. Initial applications involved polyamides (Nylon 66), but greater efficiency has been achieved with cross-linked PVP (XXI). Treatment with 1.4–2.3 kg of insoluble PVP per 100 barrels of beer provides control of chill haze and improves storage stability [12]. PVP is added after fermentation and before filtration, and it rapidly adsorbs polyphenols. Just as bentonite removes some tannins along with preferentially adsorbed protein, selective tannin adsorbents remove some proteins along with the phenolics.



Polyvinylpyrrolidone (XXI)

In addition to the adsorbents already discussed, activated charcoal and some other materials have been employed. Activated charcoal is quite reactive but it adsorbs appreciable amounts of smaller molecules (flavors, pigments) along with the larger compounds that contribute to haze formation. Tannic acid (tannin) is used to precipitate proteins, but its addition can potentially lead to the undesirable effects described previously. Other proteins with low solubility (keratin, casein, and zein) and soluble proteins (sodium caseinate, egg albumen, and serum albumin) also have selective adsorptive capacities for polyphenols, but they have not been extensively employed.

11.15 FLOUR BLEACHING AGENTS AND BREAD IMPROVERS

Freshly milled wheat flour has a pale yellow tint, and yields a sticky dough that does not handle or bake well. When the flour is stored, it slowly becomes white and undergoes an aging or maturing process that improves its baking qualities. It is a usual practice to employ chemical treatments to accelerate these natural processes [54], and to use other additives to enhance yeast leavening activity and to retard the onset of staling.

Flour bleaching involves primarily the oxidation of carotenoid pigments. This results in disruption of the conjugated double bond system of carotenoids to a less conjugated colorless system. The dough improving action of oxidizing agents is believed to involve the oxidation of sulfhydryl groups in gluten proteins. Oxidizing agents employed may participate in bleaching only, in both bleaching and dough improvement or in dough improvement only. One commonly used flour bleaching agent, benzoyl peroxide [$(C_6H_5CO)_2O_2$] exhibits a bleaching or decolorizing action, but does not influence baking properties. Materials that act both as bleaching and improving agents include chlorine gas (Cl_2), chlorine dioxide (ClO_2), nitrosyl chloride (NOCl), and oxides of nitrogen (nitrogen dioxide, NO_2, and nitrogen tetroxide (N_2O_4) [43]. These oxidizing agents are gaseous and exert their action immediately upon contact with flour. Oxidizing agents that serve primarily as dough improvers exert their action during the dough stages rather than in the flour. Included in this group are potassium bromate ($KBrO_3$), potassium iodate (KIO_3), calcium iodate [$Ca(IO_3)_2$], and calcium peroxide (CaO_2).

Benzoyl peroxide is usually added to flour (0.025–0.075%) at the mill. It is a powder and is usually added along with diluting or stabilizing agents such as calcium sulfate, magnesium carbonate, dicalcium phosphate, calcium carbonate, and sodium aluminum phosphate. Benzoyl peroxide is a free radical initiator (see Chapter 4), and it requires several hours after addition to decompose into available free radicals for initiation of carotenoid oxidation.

The gaseous agents for oxidizing flour show variable bleaching efficiencies, but effectively improve baking qualities of suitable flours. Treatment with chlorine dioxide improves flour color only slightly, but yields flour with improved dough handling properties. Chlorine gas, often containing a small amount of nitrosyl chloride, is used extensively as a bleach and improver for soft wheat cake flour. Hydrochloric acid is formed from oxidation reactions of chlorine and the resulting slightly lowered pH values lead to improved cake baking properties. Nitrogen tetroxide (N_2O_4) and other oxides of nitrogen, produced by passing air through an intense electric arc, are only moderately effective bleaching agents, but they produce good baking qualities in treated flours.

Oxidizing agents that function primarily as dough improvers can be added to flour (l0–40 ppm) at the mill. They are, however, often incorporated into a dough conditioner mix containing several inorganic salts, and then added at the bakery. Potassium bromate, an oxidizing agent used traditionally as a dough improver, remains unreactive until yeast fermentation lowers the pH of the dough sufficiently to activate it. As a result, it acts rather late in the process and causes increased loaf volume, improved loaf symmetry, and improved crumb and texture characteristics.

Early investigators proposed that the improved baking qualities resulting from treatment with oxidizing agents were attributable to inhibition of the proteolytic enzymes present in flour. However, a more recent belief is that dough improvers, at an appropriate time, oxidize sulfhydryl groups (—SH) in the gluten to yield an increased number of intermolecular disulfide bonds (—S—S—). This cross-linking would allow gluten proteins to form thin, tenacious networks of protein films that comprise the vesicles for leavening. The result is a tougher, drier, more extensible dough that gives rise to improved characteristics in the finished products. Excessive oxidation of the flour must be avoided since this leads to inferior products with gray crumb color, irregular grain, and reduced loaf volume.

The addition of a small amount of soybean flour to wheat flour intended for yeast leavened doughs has become a common practice. The addition of soybean lipoxygenase (see Chapters 4 and 6) is an excellent way to initiate the free radical oxidation of carotenoids [15]. Addition of soybean

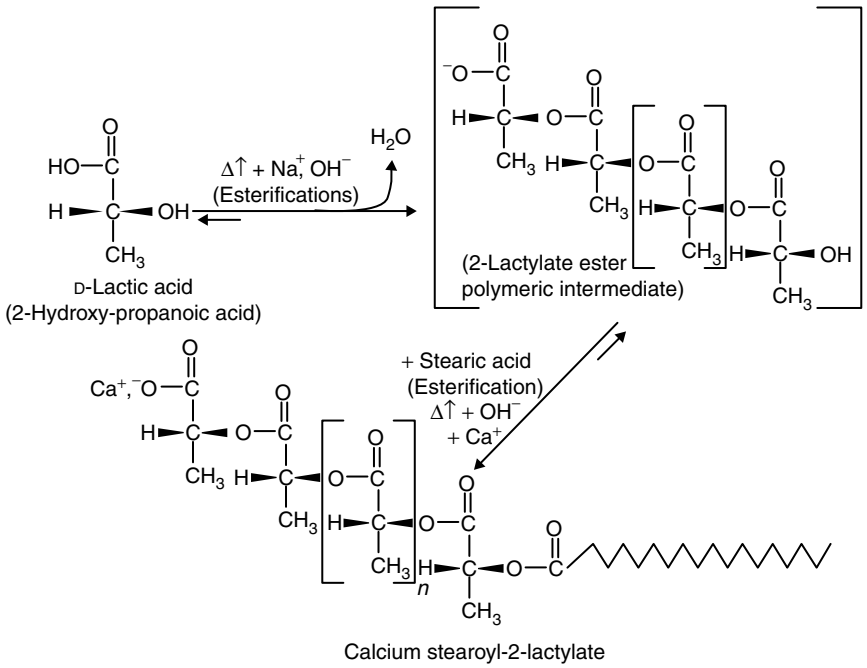


FIGURE 11.25 Reactions employed in the manufacture of steroyl-2-lactylate emulsifiers.

lipoxygenase also greatly improves the rheological properties of the dough by a mechanism not yet elucidated. While it has been suggested that lipid hydroperoxides become involved in the oxidation of gluten —SH groups, evidence indicates that other protein–lipid interactions are also involved in dough improvement by oxidants [15].

Inorganic salts incorporated into dough conditioners include ammonium chloride (NH_4Cl), ammonium sulfate [$(NH_4)_2SO_4$], calcium sulfate ($CaSO_4$), ammonium phosphate [$(NH_4)_3PO_4$], and calcium phosphate ($CaHPO_4$). They are added to dough to facilitate growth of yeast and to aid in control of pH. The principal contribution of ammonium salts is to provide a ready source of nitrogen for yeast growth. The phosphate salts apparently improve dough by buffering the pH at a slightly lower than normal value. This is especially important when water supplies are alkaline.

Other types of materials are also used as dough improvers in the baking industry. Calcium stearyoyl-2 lactylate (Figure 11.25) and similar emulsifying agents are used at low levels (up to 0.5%) to improve mixing qualities of dough and to promote increased loaf volume [56]. Hydrocolloid gums have been used in the baking industry to improve the water holding capacity of doughs and to modify other properties of doughs and baked products [34]. Carrageenan, carboxymethylcellulose, locust bean gum, and methylcellulose are among the more useful hydrocolloids in baking applications. Methylcellulose and carboxymethylcellulose have been found to retard retrogradation and staling in bread, and they also retard migration of moisture to the product surface during subsequent storage. Carrageenan (0.1%) softens the crumb texture of sweet dough products. Several hydrocolloids (e.g., carboxymethylcellulose at 0.25%) may be incorporated into doughnut mixes to significantly decrease the amount of fat absorbed during frying. This benefit apparently arises because of improvements in the dough and because a more effective hydrated barrier is established on the surface of the doughnuts.

11.16 ANTICAKING AGENTS

Several conditioning agents are used to maintain free-flowing characteristics of granular and powdered forms of foods that are hygroscopic in nature. In general, these materials function by

readily absorbing excess moisture, by coating particles to impart a degree of water repellency, and/or by providing an insoluble particulate diluent. Calcium silicate ($Ca\ SiO_3 \cdot X\ H_2O$) is used to prevent caking in baking powder (up to 5%), table salts, (up to 2%), and in other foods and food ingredients. Finely divided calcium silicate absorbs liquids in amounts up to 2.5 times its weight and still remains free flowing. In addition to absorbing water, calcium silicate also effectively absorbs oils and other nonpolar organic compounds. This characteristic makes it useful in complex powdered mixes and in certain spices that contain free essential oils.

Food grade calcium and magnesium salts of long-chain fatty acids, derived from tallow, are used as conditioning agents in dehydrated vegetable products, salt, onion, and garlic salt, and in a variety of other food ingredients and mixes that exist in powder form. Calcium stearate is often added to powdered foods to prevent agglomeration, to promote free flow during processing, and to insure freedom from caking during the shelf life of the finished product. Calcium stearate is essentially insoluble in water but adheres well to particles and provides a partial water repellent coating for the particles. Commercial stearate powders have a high bulk density (about 27 kg/m^3) and possess large surface areas that make their use as conditioners (0.5–2.5%) reasonably economical. Calcium stearate is also used as a release lubricant (1%) in the manufacture of pressed tablet-form candy.

Other anticaking agents employed in the food industry include sodium silicoaluminate, tricalcium phosphate, magnesium silicate, and magnesium carbonate. These materials are essentially insoluble in water and exhibit variable abilities to absorb moisture. Their use levels are similar to those for other anticaking agents (e.g., about 1% sodium silicoaluminate is used in powdered sugar). Microcrystalline cellulose powders are used to prevent grated or shredded cheese from clumping. Anticaking agents are either metabolized (starch, stearates) or exhibit no toxic actions at levels employed in food applications [19].

11.17 GASES AND PROPELLANTS

Gases, both reactive and inert, play important roles in the food industry. For example, hydrogen is used to hydrogenate unsaturated fats (Chapter 4), chlorine is used to bleach flour (see bleaching agents and dough improvers in this chapter) and sanitize equipment, SO_2 is used to inhibit enzymic browning in dried fruits (see sulfites and SO_2 in this chapter), ethylene gas is used to promote ripening of fruits (Chapter 17), ethylene oxide is used as a sterilant for spices (see epoxides in this chapter), and air is used to oxidize ripe olives for color development. However, the functions and properties of essentially inert gases used in food will be the topics of primary concern in the following sections.

11.17.1 Protection from Oxygen

Some processes for oxygen removal involve the use of inert gases, such as nitrogen or carbon dioxide, to flush a headspace, to strip or sparge a liquid, or to blanket a product during or after processing. Carbon dioxide is not totally without chemical influence because it is soluble in water and can lead to a tangy, carbonated taste in some foods. The ability of carbon dioxide to provide a dense, heavier-than-air, gaseous blanket over a product makes it attractive in many processing applications. Nitrogen blanketing requires thorough flushing followed by a slight positive pressure to prevent rapid diffusion of air into the system. A product that is thoroughly evacuated, flushed with nitrogen, and hermetically sealed will exhibit increased stability against oxidative deterioration [30].

11.17.2 Carbonation

The addition of carbon dioxide (carbonation) to liquid products, such as carbonated soft drinks, beer, some wines, and certain fruit juices causes them to become effervescent, tangy, slightly tart, and somewhat tactual. The quantity of carbon dioxide used and the method of introduction varies

widely with the type of product [30]. For example, beer becomes partially carbonated during the fermentation process, but is further carbonated before bottling. Beer usually contains 3–4 volumes of carbon dioxide (1 volume of beer at 16°C and 1 atmosphere pressure contains 3–4 volumes of carbon dioxide gas at the same temperature and pressure). Carbonation is often carried out at lowered temperatures (4°C) and elevated pressures to increase carbon dioxide solubility. Other carbonated beverages contain from 1.0 to 318 volumes of carbon dioxide depending upon the effect desired. The retention of large amounts of carbon dioxide in solutions at atmospheric pressure have been ascribed to surface adsorption by colloids and to chemical binding. It is well established that carbamino compounds are formed in some products by rapid, reversible reactions between carbon dioxide and free amino groups of amino acids and proteins. In addition, formation of carbonic acid (H_2CO_3) and bicarbonate ions (HCO_3^-) also aid in stabilizing the carbon dioxide system. Spontaneous release of carbon dioxide from beer, that is, gushing, has been associated with trace metallic impurities and with the presence of oxalate crystals that provide nuclei for nucleation of gas bubbles.

11.17.3 Propellants

Some fluid food products are dispensed as liquids, foams, or sprays from pressurized aerosol containers. Since the propellant usually comes into intimate contact with the food, it becomes an incidental food component or ingredient. The principal propellants for pressure dispensing of foods are nitrous oxide, nitrogen, and carbon dioxide [30]. Foam and spray type products are usually dispensed by nitrous oxide and carbon dioxide because these propellants are quite soluble in water and their expansion during dispensing assists in the formation of the spray or foam. Carbon dioxide is also employed for products such as cheese spreads where tanginess and tartness are acceptable characteristics. Nitrogen, because of its low solubility in water and fats, is used to dispense liquid streams in which foaming should be avoided (catsup, edible oils, syrups). The use of all of these gases in foods is regulated, and the pressure must not exceed 100 psig at 21°C or 135 psig at 54°C. At these conditions, none of the gases liquify and a large portion of the container is occupied by the propellant. Thus as the product is dispensed, the pressure drops and this can lead to difficulties with product uniformity and completeness of dispensing. The gaseous propellants are nontoxic, nonflammable, economical, and usually do not cause objectionable color or flavors. However, carbon dioxide, when used alone, imparts an undesirable taste to some foods.

Liquid propellants also have been developed and approved for food use, but environmental concerns regarding ozone depletion in the upper atmosphere has led to the banning of the chlorofluorocarbon substances. Those approved for foods were octafluorocyclobutane or Freon C-318 ($CF_2—CF_2—CF_2—CF_2$) and chloropentafluoroethane or Freon 115 ($CClF_2—CF_3$). Although potentially hazardous, the flammable hydrocarbons propane, butane, and isobutene are used as propellant gases for vegetable-oil-based aerosol and water-based emulsion cooking sprays. When used, a portion of the propellants exist in the container as a liquid layer situated on top of the food product, and an appropriate headspace containing vaporized propellant is also present. Use of a liquified propellant enables dispensing to occur at a constant pressure, but the contents must be shaken first to provide an emulsion that will efficiently foam or spray upon discharge from the container. Constant pressure dispensing is essential for good performance of spray-type aerosols. These propellants are nontoxic at levels encountered and they do not impart off-flavors to foods. They give particularly good foams because they are highly soluble in any fat that may be present, and they can be effectively emulsified.

11.18 SUMMARY

Summarized in Table 11.5 [9,16,17,21,28,36] are various kinds of food additives and their functions in food.

TABLE 11.5
Selected Food Additives[a]

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
I. Processing additives			
Aerating and foaming agents	Carbon dioxide	Carbonation, foaming	11
	Nitrogen	Foaming	11
	Sodium bicarbonate	Foaming	11
Antifoam agents	Aluminum stearate	Yeast processing	—
	Ammonium stearate	Beet sugar processing	—
	Butyl stearate	Beet sugar, yeast	—
	Decanoic acid	Beet sugar, yeast	—
	Dimethylpolysiloxane	General use	—
	Dimethylpolysilicone	General use	—
	Lauric acid	Beet sugar, yeast	—
	Mineral oil	Beet sugar, yeast	—
	Oleic acid	General use	—
	Oxystearin	Beet sugar, yeast	—
	Palmitic acid	Beet sugar, yeast	—
	Petroleum waxes	Beet sugar, yeast	—
	Silicone dioxide	General use	—
	Stearic acid	Beet sugar, yeast	—
Catalysts (including enzymes)	Nickel	Lipid hydrogenation	4
	Amylase	Starch conversion	3,6
	Glucose oxidase	Oxygen scavenger	6
	Lipase	Dairy flavor developer	6
	Papain	Chill-proofing beer	6
	Pepsin	Meat tenderizer	6
	Rennin	Cheese production	6
Clarifying and flocculating agents	Bentonite	Absorbs proteins	11
	Gelatin	Complexes polyphenols	11
	Polyvinylpyrrolidone	Complexes polyphenols	11
	Tannic acid	Complexes proteins	11
Color control agents	Ferrous gluconate	Dark olives	—
	Magnesium chloride	Canned peas	9
	Nitrate, nitrite (potassium, sodium)	Cured meat	9,11
	Sodium erythorbate	Cured meat color	9
Freezing and cooling agents	Carbon dioxide	—	11
	Liquid nitrogen	—	—
Malting and fermenting aids	Ammonium chloride	Yeast nutrients	11
	Ammonium phosphate	—	—
	Ammonium sulfate	—	—
	Calcium carbonate	—	—
	Calcium phosphate	—	—
	Calcium phosphate (dibasic)	—	—
	Calcium sulfate	—	—
	Potassium chloride	—	—
	Potassium phosphate	—	—
Material handling aids	Aluminum phosphate	Anticaking, free flow	11
	Calcium silicate	Anticaking, free flow	11
	Calcium stearate	Anticaking, free flow	11

(Continued)

TABLE 11.5 (Continued)

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
	Dicalcium phosphate	Anticaking, free flow	11
	Dimagnesium phosphate	Anticaking, free flow	11
	Kaolin	Anticaking, free flow	11
	Magnesium silicate	Anticaking, free flow	—
	Magnesium stearate	Anticaking, free flow	—
	Sodium carboxymethylcellulose	Bodying, bulking	11
	Sodium silicoaluminate	Anticaking, free flow	11
	Starches	Anticaking, free flow	3
	Tricalcium phosphate	Anticaking, free flow	11
	Tricalcium silicate	Anticaking, free flow	12
	Xanthan (other gums)	Bodying, bulking	3,11
Oxidizing agents	Acetone peroxides	Free radical initiator	11
	Benzoyl peroxide	Free radical initiator	11
	Calcium peroxide	Free radical initiator	11
	Hydrogen peroxide	Free radical initiator	—
	Sulfur dioxide	Dried fruit bleach	11
pH control and modification agents			
Acidulants (acids)	Acetic acid	Antimicrobial agent	11
	Citric acid	Chelating agent	4,11
	Fumaric acid	Antimicrobial agent	11
	δ-Gluconolactone	Leavening agent	11
	Hydrochloric acid	—	11
	Lactic acid	—	11
	Malic acid	Chelating agent	11
	Phosphoric acid	—	11
	Potassium acid tartrate	Leavening agent	11
	Succinic acid	Chelating agent	11
	Tartaric acid	Chelating agent	11
Alkalies (bases)	Ammonium bicarbonate	Carbon dioxide source	11
	Ammonium hydroxide	—	—
	Calcium carbonate	—	—
	Magnesium carbonate	—	—
	Potassium carbonate	Carbon dioxide source	—
	Potassium hydroxide	—	—
	Sodium bicarbonate	Carbon dioxide source	11
	Sodium carbonate	—	—
	Sodium citrate	Emulsifier salt	11
	Trisodium citrate	Emulsifier salt	11
Buffering salts	Ammonium phosphate (mono, dibasic)	—	11
	Calcium citrate	—	—
	Calcium gluconate	—	—
	Calcium phosphate (mono, dibasic)	—	—
	Potassium acid tartrate	—	—
	Potassium citrate	—	—
	Potassium phosphate (mono, dibasic)	—	—
	Sodium acetate	—	—
	Sodium acid pyrophospate	—	—
	Sodium citrate	—	—

TABLE 11.5 (Continued)

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
	Sodium phosphate (mono, di, tribasic)	—	—
	Sodium potassium tartrate	—	—
Release and antistick agents	Acylated monoacylglycerols	—	4,11
	Beeswax	—	4,11
	Calcium stearate	—	4,11
	Magnesium silicate	—	4,11
	Mineral oil	—	—
	Mono- and diacylglycerols	Emulsifiers	4
	Starches	—	3
	Stearic acid	—	4,11
	Talc	—	—
Sanitizing and fumigating agents	Chlorine	Oxidant	—
	Methyl bromide	Insect fumigant	—
	Sodium hypochlorite	Oxidant	—
Separation and filtration aids	Diatomaceous earth	—	—
	Ion-exchange resins	—	—
	Magnesium silicate	—	—
Solvents, carriers, and encapsulating agents	Acetone	Solvent	—
	Agar–agar	Encapsulation	3
	Arabinogalactan	Encapsulation	3
	Cellulose	Carrier	3
	Glycerine	Solvent	4,11
	Guar gum	Encapsulation	3
	Methylene chloride	Solvent	—
	Propylene glycol	Solvent	11
	Triethyl citrate	Solvent	—
Washing and surface removal agents	Sodium dodecylbenzene-sulfonate	Detergent	—
	Sodium hydroxide	Lye peeling	—
II. Final product additives			
Antimicrobial agents	Acetic acid (and salts)	Bacteria, yeast	11
	Benzoic acid (and salts)	Bacteria, yeast	11
	Ethylene oxide	General sterilant	11
	p-Hydroxybenzoate alkyl esters	Molds, yeast	11
	Nitrates, nitrites (K, Na)	*C. botulinum*	9,11
	Propionic acid (and salts)	Mold	11
	Propylene oxide	General sterilant	11
	Sorbic acid (and salts)	Mold, yeast, bacteria	11
	Sulfur dioxide and sulfites	General	11
Antioxidants	Ascorbic acid (and salts)	Reducing agent	4,7
	Ascorbyl palmitate	Reducing agent	11
	BHA	Free radical terminator	4,11
	BHT	Free radical terminator	4,11
	Gum guaiac	Free radical terminator	4,11
	Propylgallate	Free radical terminator	4,11
	Sulfite and metabisulfite salts	Reducing agents	4,11
	Thiodipropionic acid (and esters)	Peracid decomposer	11

(Continued)

TABLE 11.5 (Continued)

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
Appearance control agents			
Colors and color modifiers	Annatto	Cheese, butter, baked goods	9
	Beet powder	Frosting, soft drinks	9
	Caramel	Confectionary	9
	Carotene	Margarine	9
	Cochineal extract	Beverages	9
	FD&C No. 3	Mint Jelly, beverages	9
	FD&C No. 3 (erythrosine)	Canned fruit cocktail	9
	Titanium dioxide	White candy, Italian cheeses	9
	Turmeric	Pickles, sauces	9
Other appearance agents	Beeswax	Gloss, polish	4
	Glycerine	Gloss, polish	4
	Oleic acid	Gloss, polish	4
	Sucrose	Crystalline glaze	3
	Wax, caranuba	Gloss, polish	—
Flavors and flavor modifiers			
Flavoring agents[b]	Essential oils	General	10
	Herbs and spices	General	10
	Plant extractives	General	10
	Synthetic flavor compounds	General	—
Flavor potentiators	Disodium guanylate	Meats and vegetables	10
	Disodium inosinate	Meats and vegetables	10
	Maltol	Bakery goods, sweets	10
	Monosodium glutamate	Meats and vegetables	10
	Sodium chloride	General	—
Moisture control agents	Glycerine	Plasticizer, humectant	3,10
	Gum acacia	—	—
	Invert sugar	—	3
	Propylene glycol	—	10
	Mannitol	—	3,10
	Sorbitol	—	3,10
Nutrient, dietary supplements			
Amino acids	Alanine	—	5
	Arginine	Essential	5
	Aspartic acid	—	5
	Cysteine	—	5
	Cystine	—	5
	Glutamic acid	—	5
	Histidine	—	5
	Isoleucine	Essential	5
	Leucine	Essential	5
	Lysine	Essential	5
	Methionine	Essential	5
	Phenylalanine	Essential	5
	Proline	—	5
	Threonine	Essential	5
	Valine	Essential	5

TABLE 11.5 (Continued)

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
Minerals	Boric acid	Boron source	8
	Calcium carbonate	Breakfast cereals	8
	Calcium citrate	Cornmeal	8
	Calcium phosphates	Enriched flour	8
	Calcium pyrophosphate	Enriched flour	8
	Calcium sulfate	Bread	8
	Cobalt carbonate	Cobalt source	8
	Cobalt chloride	Cobalt source	8
	Cupric chloride	Copper source	8
	Cupric gluconate	Copper source	8
	Cupric oxide	Copper source	8
	Calcium fluoride	Water fluoridations	—
	Ferric phosphate	Iron source	8
	Ferric pyrophosphate	Iron source	8
	Ferrous gluconate	Iron source	8
	Ferrous sulfate	Iron source	8
	Iodine	Iodine source	8
	Iodide, cuprous	Table salt	8
	Iodate, potassium	Iodine source	8
	Magnesium chloride	Magnesium source	8
	Magnesium oxide	Magnesium source	8
	Magnesium phosphates	Magnesium source	8
	Magnesium sulfate	Magnesium source	8
	Manganese citrate	Manganese source	8
	Manganese oxide	Manganese source	8
	Molybdate, ammonium	Molybdenum source	8
	Nickel sulfate	Nickel source	8
	Phosphates, calcium	Phosphorous source	8
	Phosphates, sodium	Phosphorous source	8
	Potassium chloride	NaCl substitute	—
	Zinc chloride	Zinc source	8
	Zinc stearate	Zinc source	8
Vitamins	*p*-Aminobenzoic acid	B complex factor	7
	Biotin	—	7
	Carotene	Provitamin A	7
	Folic acid	—	7
	Niacin	—	7
	Niacinamine	Enriched flour	7
	Pantothenate, calcium	B complex vitamin	7
	Pyridoxine hydrochloride	B complex vitamin	7
	Riboflavin	B complex vitamin	7
	Thiamine hydrochloride	Vitamin B_1	7
	Tocopherol acetate	Vitamin E	7
	Vitamin A acetate	—	—
	Vitamin B_{12}	—	7
	Vitamin D	—	7

(Continued)

TABLE 11.5 (Continued)

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
Miscellaneous nutrients	Betaine hydrochloride	Dietary supplement	7
	Choline chloride	Dietary supplement	7
	Inositol	Dietary supplement	7
	Linoleic acid	Essential fatty acid	4
	Rutin	Dietary supplement	7
Sequestrants (chelating agents)	Calcium citrate	—	11
	Calcium disodium EDTA	—	11
	Calcium gluconate	—	—
	Calcium phosphate (monobasic)	—	—
	Citric acid	—	11
	Disodium EDTA	—	11
	Phosphoric acid	—	11
	Potassium citrate	—	—
	Potassium phosphate (mono, dibasic)	—	—
	Sodium acid pyrophosphate	—	8,11
	Sodium citrate	—	11
	Sodium gluconate	—	—
	Sodium hexametaphosphate	—	—
	Sodium phosphate (mono, di, tri)	—	—
	Sodium potassium tartrate	—	—
	Sodium tartrate	—	—
	Sodium tripolyphosphate	—	8,11
	Tartaric acid	—	—
Specific gravity control agent	Brominated vegetable oil	Increase density of oil droplets	11
Surface tension control agents	Dioctyl sodium sulfosuccinate	—	—
	Ox bile extract	—	—
	Sodium phosphate (dibasic)	—	—
Sweeteners			
Non-nutritive	Acesulfame K	—	10,11
	Ammonium saccharin	—	10,11
	Calcium saccharin	—	10,11
	Saccharin	—	10,11
	Sodium saccharin	—	10,11
Nutritive	Aspartame	—	10,11
	Glucose	—	3
	Sorbitol	—	3,11
Texture and consistency control agents			
Emulsifiers and emulsifier salts	Calcium stearoyl-2-lactylate	Dried egg white, bakery	4,11
	Cholic acid	Dried egg white	4
	Desoxycholic	Dried egg white	4
	Dioctyl sodium sulfosuccinate	General	—
	Fatty acids (C_{10}—C_{18})	General	4
	Lactylic esters of fatty acids	Shortening	4,11
	Lecithin	General	4
	Mono- and diacylglycerides	General	4

TABLE 11.5 (Continued)

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
	Ox bile extract	General	—
	Polyglycerol esters	General	11
	Polyoxyethylene sorbitan esters	General	4,11
	Propyleneglycol, mono, diesters	General	4
	Potassium phosphate, tribasic	Processed cheese	11
	Potassium polymetabisulfite		—
	Potassium pyrophosphate	Processed cheese	11
	Sodium aluminum sulfate	General	11
	Sodium citrate	Processed cheese	11
	Sodium metaphosphate	General	11
	Sodium phosphate, dibasic	Processed cheese	11
	Sodium phosphate, monobasic	Processed cheese	11
	Sodium phosphate, tribasic	Processed cheese	11
	Sodium pyrophosphate	General	11
	Sorbitan monooleate	Dietary products	4
	Sorbitan monopalmitate	Flavor dispersion	4
	Sorbitan monostearate	General	4
	Sorbitan tristearate	Confection coatings	4
	Stearoyl-2-lactylate	Bakery shortening	4
	Stearoyl monoglyceridyl citrate	Shortenings	4
	Taurocholic acid	Egg whites	4
Firming agents	Aluminum sulfates (Alum)	Pickles	11
	Calcium carbonate	General	11
	Calcium chloride	Canned tomatoes	11
	Calcium citrate	Canned tomatoes	11
	Calcium gluconate	Apple slices	11
	Calcium hydroxide	Fruit slices	11
	Calcium lactate	Apple slices	11
	Calcium phosphate, monobasic	Canned tomatoes	11
	Calcium sulfate	Canned potatoes, tomatoes	11
	Magnesium chloride	Canned peas	9
Leavening agents	Ammonium bicarbonate	Carbon dioxide source	11
	Ammonium phosphate (dibasic)	—	11
	Calcium phosphate	—	11
	δ-Gluconolactone	—	11
	Sodium acid pyrophosphate	—	11
	Sodium aluminum phosphate	—	11
	Sodium aluminum sulfate	—	11
	Sodium bicarbonate	Carbon dioxide source	11
Masticatory substances	Paraffin (synthetic)	Chewing gum base	11
	Pentaerythritol ester of rosin	Chewing gum base	11
Propellants	Carbon dioxide	—	11
	Nitrous oxide	—	11
Stabilizers and thickeners	Acacia gum	Foam stabilizer	3,11
	Agar	Ice cream	3,11
	Alginic acid	Ice cream	3,11
	Carrageenan	Chocolate drinks	3,11

(Continued)

TABLE 11.5 (Continued)

Class and General Function	Chemical Name	Additional or More Specific Function	Source of Discussion (Chapter)
	Guar gum	Cheese foods	3,11
	Hydroxypropyl-methylcellulose	General	3,11
	Locust bean gum	Salad dressing	3,11
	Methyl cellulose	General	3,11
	Pectin	Jellies	3,11
	Sodium carboxymethylcellulose	Ice cream	3,11
	Tragacanth gum	Salad dressing	3,11
Texturizers	Carrageenan	General	3,11
	Mannitol	—	3,11
	Pectin	—	3,11
	Sodium caseinate	—	5
	Sodium citrate	—	11
Tracers	Titanium dioxide	Vegetable protein extenders	12

[a] For additional information see References 9, 16, 17, 21, 28, and 36; also see General Reference list.

[b] Individual members comprising flavoring agents are too numerous to mention. See References 21 and 28 for comprehensive listings; also see General Reference list.

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12 Bioactive Substances: Nutraceuticals and Toxicants

Chi-Tang Ho, Mohamed M. Rafi, and Geetha Ghai

CONTENTS

Bioactive substances are classified as nutraceuticals and toxicants. Nutraceuticals are naturally derived, bioactive compounds that have health-promoting, disease-preventing, and/or medicinal properties and have an impact on human health. Toxicants are also naturally occurring or process-induced chemical compounds that have adverse effects on human health. Fruits, vegetables, common beverages, grains, nuts, oils, marine products, medicinal plants, and herbal products all contain both nutraceuticals and toxicants. Depending on the source, the amount of bioactives that possess diversified pharmacological properties will vary. If the source has a greater quantity of nutraceuticals than toxicants, that particular source will have the potential to provide protection from chronic diseases. Most fruits and vegetables that are consumed fall in this category.

12.1 REGULATORY STANDPOINT

The use of bioactives in alternative medicine and dietary supplements are very popular today, even though their efficacy and safety have not been scientifically confirmed. In 1994, the U.S. Congress passed the Dietary Supplement Health and Education Act (DSHEA), which resulted in significantly

reduced requirements for labeling dietary supplements compared with drugs and food additives. This act substantially weakened the authority of the U.S. Food and Drug Administration (FDA) to ensure regulation on safety, purity, and efficacy of dietary supplements. The FDA has not issued any final regulations on nutraceuticals or medicinal foods and, in fact, dietary supplements are not legally considered a food or a drug [23].

12.2 HEALTH STANDPOINT

Herbs and medicinal plants have been used for centuries to treat many diseases throughout the world and 80% of the world population relies on botanical preparations as medicine for their health needs. The biological activity of a natural product is very often believed to be the result of the combined action of several of its constituents. However in most cases, the active ingredient of the natural product has not yet been completely characterized. Approximately 15 million Americans take herbs at the same time as prescription medications.

Benefits to health by eating more fruits and vegetables initially came from observing populations, called observational epidemiologic studies. Nutrition-related observations of populations are referred to as nutritional epidemiology. Most often, nutritional epidemiology examines disease relationship against a single or a few nutrients or foods. Conceptual and methodological limitations have been attributed to nutritional epidemiological methods, as people do not eat single nutrients or foods or well-defined mixtures of them; instead, we eat a variety of foods with many nutrients. Recently, complementary methods have been developed to analyze dietary patterns to overcome these limitations [27]. Although solutions for these limitations are being sought, we should bear in mind that valuable information has been obtained using such nutritional epidemiological methods. A successful example is that of vitamins in human nutrition.

Nutritional epidemiological studies have shown that environmental factors, especially food components, have a major impact on hormone-related cancer prevention and a low intake of fruits and vegetables is associated with high mortality in cardiovascular diseases (CVDs) [53]. One class of substances indicated as being responsible for these cancer-protective effects are phytochemicals and phytoestrogens, which are abundant in soy products [38]. Many herbs, foods, and spices contain flavonoids, phytoestrogens, and unidentified phytochemicals that exhibit estrogenic activity* in prostate cancer patients. Chemoprevention through the consumption of nutraceuticals, for example, resveratrol from grapes, lycopene from tomatoes, and genistein from soy products may reduce both morbidity and mortality from cancer [38]. The foods and herbs that possess anticancer activity include garlic, soybeans, cabbage, ginger, licorice, onions, flax, turmeric, cruciferous vegetables, tomatoes, peppers, brown rice, wheat, and the umbelliferous vegetables such as carrots, celery, and parsley [38]. Natural products and their isolated constituents have been shown to possess strong chemopreventive activity in animal models [38]. The effect of nutraceuticals on apoptotic† pathways, signaling pathways,‡ and/or different targets in cancer would be helpful in the design and development of novel cancer-preventive agents.

Laboratory research is conducted using well-characterized model systems outside and inside test organisms. *In vitro* assays are conducted outside the organism in an artificial environment that simulates conditions within the organism. These include studies using isolated enzymes, cell cultures, tissues, and organs. *In vivo* systems are usually based on observing a relevant physiological response or pathology of a living organism to a treatment. Specific models are developed for different disease states and the biological materials can either be obtained from specialized vendors or developed

* "Estrogenic" compounds may mimic, supplement, or antagonize estrogen and related hormone action in organisms.

† Apoptosis is an orderly or programmed cellular death, a normal feature of cell life cycle, biological turnover, and growth and development. Necrosis is cellular death or turnover often caused by injury.

‡ A chain of biochemical events that prompts cellular responses to a stimulus.

in the investigators' laboratories. Many techniques are used to generate these models; for example, models can be developed by using chemicals or breeding techniques. In the past decade, genetic engineering has created many valuable mouse models called knockout mice.* Laboratory research is conducted using these models to understand the disease process and how to cure it. Today, the same *in vitro* and *in vivo* models are being used to find ways to prevent chronic diseases. Although there may be limitations to laboratory research, most often long-term research provides sound scientific information, which can either be translated to human studies or in support of epidemiological observations.

12.3 HEALTH-PROMOTING PHYTOCHEMICALS

Many phytochemicals in fruits and vegetables have been isolated and have demonstrated health-promoting properties. They can be categorized into several classes.

12.3.1 Carotenoids

Carotenoids are natural, fat-soluble pigments that provide bright coloration to plants and animals. They also act as antioxidants, and some of them possess vitamin A activity. One defining characteristic of carotenoids is the chemical structure of their backbone molecule, a 40-carbon polyene chain, derived from isoprene. The polyene backbone consists of conjugated double bonds, which allows the carotenoids to take up excess energy from other molecules through a nonradiative energy transfer mechanism [45]. This characteristic may be responsible for the antioxidant activity seen in biological carotenoids, a major role that is to quench singlet oxygen. In addition to scavenging reactive oxygen and free radicals, other health benefits related to this observed antioxidative activity include enhancement of immune function, protection from sunburn, and inhibition of the development of certain types of cancers [6].

Carotenoids are sensitive to light, air, excessive heat, and acid exposure. This sensitivity leads to vulnerabilities during processing and storage, and care should be taken to minimize carotenoid losses.

β-Carotene (Figure 12.1) is the most common carotenoid in food and the most potent of the provitamin A carotenoids. β-Carotene is known for its many health-promoting characteristics such as enhancement of the immune system, by improved activity and changes in immune cell numbers, and decreased risk of degenerative diseases such as cancer, CVDs, age-related macular degeneration, and cataract formation [35]. β-Carotene is primarily found in red palm oil, palm fruits, leafy green vegetables, carrots, sweet potatoes, mature squashes, pumpkin, mangoes, and papayas.

Epidemiological and animal studies support the hypothesis that β-carotene can prevent cancer in humans [68]. On the contrary, chemoprevention trials with β-carotene either alone or in combination with vitamin A or vitamin E actually increased the incidence of lung cancer in high-risk groups of humans [44,47]. Other *in vitro* and *in vivo* studies have shown that while β-carotene itself may be anticarcinogenic, its oxidized products may facilitate carcinogenesis, thus providing an explanation to the chemoprevention trials. In other words, the instability of β-carotene molecule in a free-radical-rich environment, the lungs of cigarette smokers, for example, may generate oxidized carotene products that are actually carcinogens [68].

Additionally, β-carotene is believed to have antioxidant activity. It has been shown to exhibit radical-trapping behavior only at partial pressures of oxygen substantially less than that in normal air [7]. Such low oxygen partial pressures are found in most tissues under physiological conditions. At higher oxygen pressure it loses the antioxidant activity and shows a pro-oxidant effect

* A genetically engineered mouse where one or more genes are rendered inoperable.

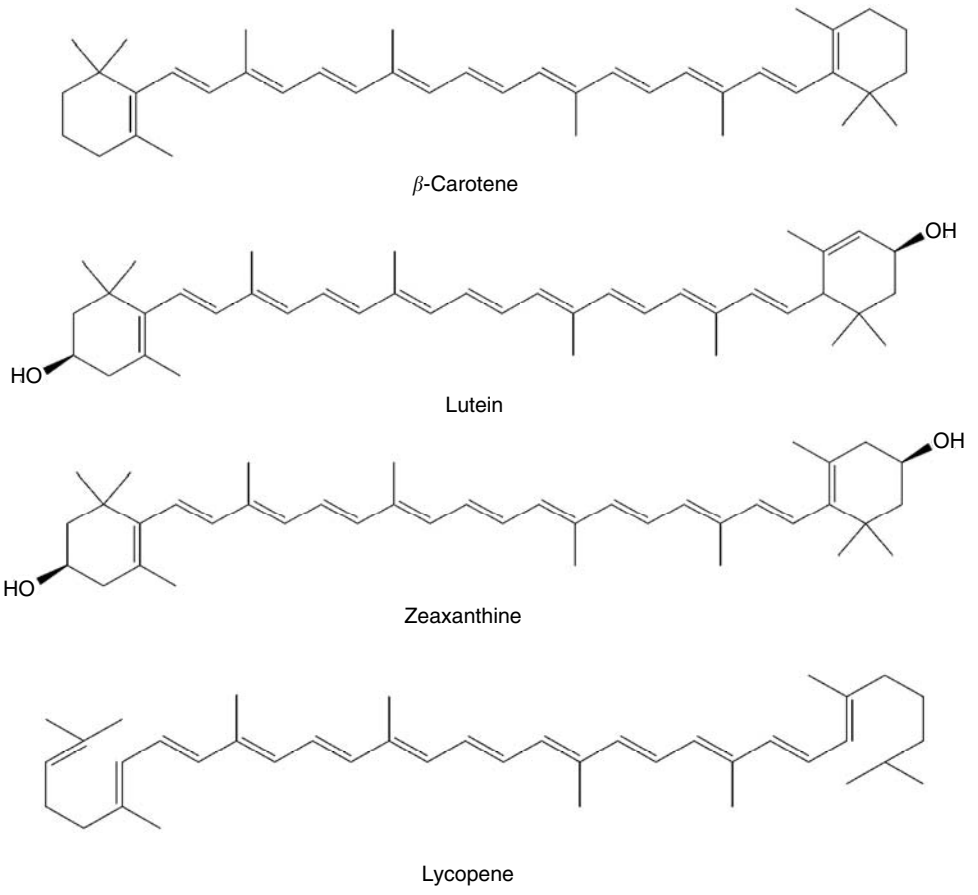


FIGURE 12.1 Structures of β-carotene, lutein, zeaxanthine, and lycopene.

[7]. These studies clearly emphasize the need to understand the chemistry of specific nutraceuticals. If biological research is conducted without consideration of the chemistry of a molecule like β-carotene, a conundrum of results such as with the β-carotene trial will be generated, adding more confusion to the field of bioactives.

Lutein and its isomer, zeaxanthin, are yellow pigments that belong to the classes of nonprovitamin A carotenoids (Figure 12.1). Unlike other carotenoids, hydroxyl groups are substituted on the ring structures at the end of the conjugated double bond chains of lutein and zeaxanthin; therefore, they are also called oxycarotenoids or xanthophylls. Lutein is naturally occurring and found predominantly in dark green, leafy vegetables such as spinach and kale. Zeaxanthin gives corn its yellow color. Other food sources include squash, peas, cabbage, peppers, oranges, kiwis, and grapes. Although there is no sufficient evidence to support any health claims of a relationship between lutein or zeaxanthin and any chronic disease, there is a growing body of evidence (including *in vivo*, *in vitro*, and epidemiological studies) supporting the claim that lutein and zeaxanthin contribute to health and delay age-related macular degeneration of the eyes and, to a lesser extent, cancers and heart diseases [10]. The evidence for the role of lutein and zeaxanthin in eye health is the strongest because of their exclusive presence in the ocular tissues and the high numbers of epidemiological studies that have been conducted on this subject. With a high accumulation in the macula of the eye, the area of highest visual acuity, lutein and zeaxanthin are proposed to have the ability to filter out harmful blue light, while at the same time acting as antioxidants to quench potentially damaging reactive oxygen species (ROS) [60].

Lycopene (Figure 12.1), a carotenoid found in tomatoes, watermelon, papaya, apricot, and orange and pink grapefruit, has antioxidant and anticancer activities. About 80% of dietary lycopene is from tomatoes and tomato-related products, with watermelon being a secondary source. The bioavailability of lycopene is rather poor, but is improved by thermal processing [61]. Numerous studies have suggested reduced risk of prostate cancer from the consumption of processed tomato products. Consistently, a lower risk of a variety of cancers and CVD has been associated with higher consumption of tomato-based products. Although these beneficial health effects of lycopene are thought to be due to its antioxidant properties, evidence is accumulating to suggest other mechanisms of action like hormone and immune system modulation [54]. Lycopene is the most abundant carotenoid in human plasma, which may imply its elevated level of importance in the human body compared with other carotenoids, such as β-carotene and lutein. In human serum, lycopene is generally bound to low-density lipoprotein (LDL), and transported to various tissue sites, such as liver, adrenal, testes, and prostate [54]. Additionally, in the human body, lycopene is a prevailing carotenoid, with high levels found in testes, adrenal glands, liver, and prostate.

12.3.2 Flavonoids

Flavonoids are ubiquitous in plants; almost, all plant tissues are able to synthesize flavonoids. There are also a wide variety of types—at least 2,000 naturally occurring flavonoids. Flavonoids are present in edible fruits, leafy vegetables, roots, tubers, bulbs, herbs, spices, legumes, tea, coffee, and red wine. They can be classified into seven groups: flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols (catechins), and anthocyanidins. The structures of these flavonoids are given in Figure 12.2. Examples of common flavonoids in foods are listed in Table 12.1. In general, the leaves, flowers, and fruits or the plant itself contain flavonoid glycosides, woody tissues contain aglycones, and seeds may contain both.

As a result of their ubiquity in plants, flavonoids are an integral part of the human diet. It is estimated [28] that the average American's daily intake of flavonoids is close to 1 g per person. However, this may be an overestimate of flavonoid intake when compared to the results of the Zutphen Elderly Study by Hertog et al. [24]. They measured the content of selected flavonoids, namely quercetin, kaempferol, myricetin, apigenin, and luteolin, in various foods most commonly consumed in The Netherlands. By assessing the flavonoid intake of 805 men aged 65–84 years, they found that the mean baseline flavonoid intake was 25.9 mg/day. Certain later studies provided more precise individual data concerning the intake of various classes of flavonoids. For example, the consumption of flavonols for Americans has been estimated at ~20–25 mg/day [37].

Almost all flavonoids possess several common biological and chemical properties: (1) antioxidant activity, (2) the ability to scavenge active oxygen species, (3) the ability to scavenge electrophiles, (4) the ability to inhibit nitrosation, (5) the ability to chelate metals (such as Fe and Cu), (6) the potential to produce hydrogen peroxide in the presence of certain metals, and (7) the capability to modulate certain cellular enzyme activities [28]. It appears that diets rich in flavonoids may protect against CVDs, neurodegenerative disorders, and some forms of cancer.

Among the most well-studied health-promoting flavonoids in recent years are green tea catechins. Tea is one of the most widely consumed beverages in the world; it has been used for medicinal purposes in China and Japan for thousands of years. More than 300 different kinds of tea are produced from the leaves of *Camellia sinensis* by different manufacturing processes. Generally they are divided into three types: green tea (nonfermented), oolong tea (semifermented), and black tea (fermented). Green tea and oolong tea are more popular in China, Japan, Korea, and some African countries, whereas black tea is preferred in India and Western countries. Experimental and epidemiological studies have linked the consumption of tea to reduced risk of CVD and cancer. These effects have been attributed to the polyphenolic compounds in tea [55]. Catechins are the most abundant polyphenols in green tea. A typical cup of brewed green tea contains, by dry weight, 30–40% catechins including epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate

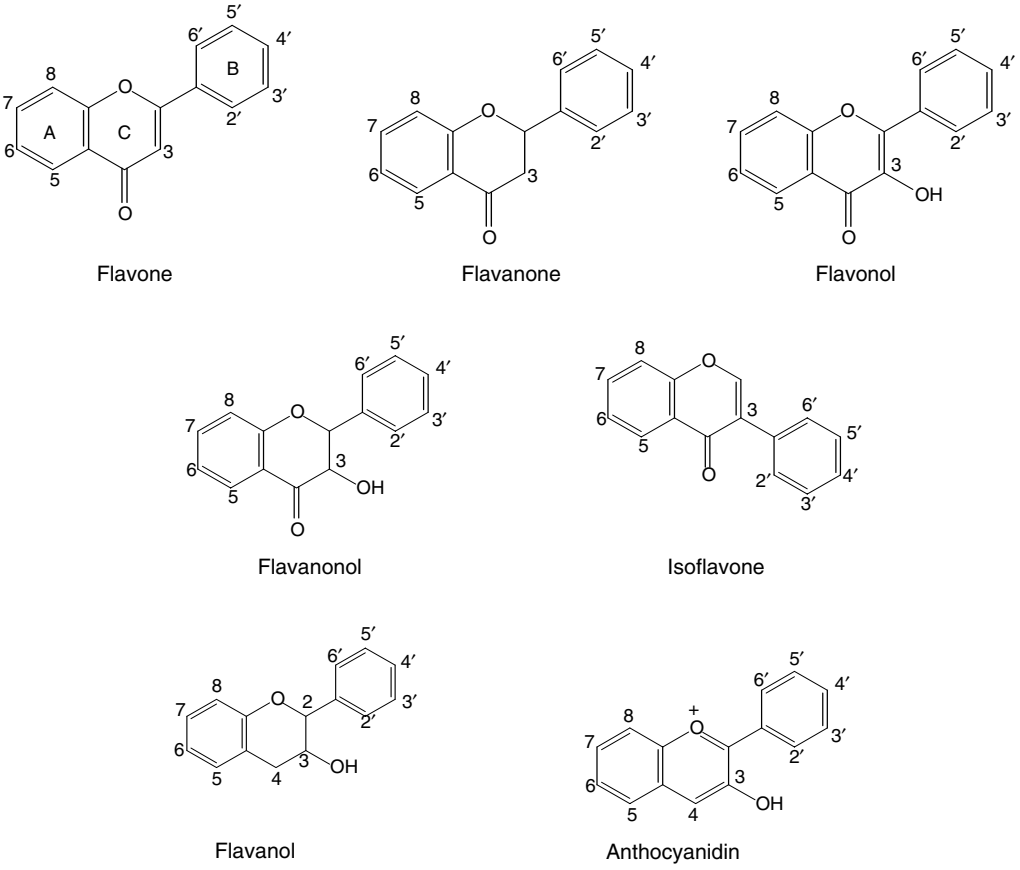
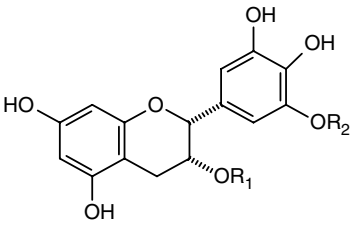


FIGURE 12.2 Structures of flavonoids.

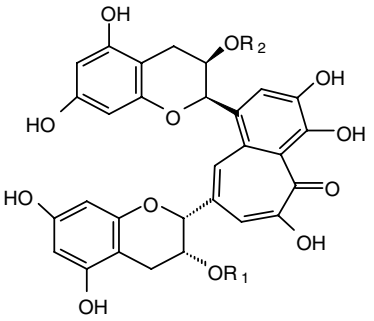
TABLE 12.1
Different Classes of Flavonoids, Their Substitution Patterns and Dietary Sources

Class	Name	Substitution	Dietary Source
Flavone	Apigenin	5,7-OH	Parsley, celery
	Rutin	5,7,3′,4′-OH,3-*O*-rutinose	Buckwheat, citrus
	Tangeretin	4,5,6,7,4′-OCH_3	Citrus
Flavanone	Naringin	5,4′-OH	Citrus
	Naringenin	5,7,4′-OH	Orange peel
Flavonol	Kaempferol	3,5,7,4′-OH	Broccoli, tea
	Quercetin	3,5,7,3′,4′-OH	Onion, broccoli, apples, berries
Flavanonol	Taxifolin	3,5,7,3′,4′-OH	Fruits
Isoflavone	Genistein	5,7,4′-OH	Soybean
	Daidzein	4′-OH, 7-*O*-glucose	Soybean
	Puerarin	7,4′-OH, 8-*C*-glucose	Kudzu
Flavanol (catechin)	(−)-Epicatechin	3,5,7,3′,4′-OH	Tea
	(−)-Epigallocatechin	3,5,7,3′,4′ ,5′-OH	Tea
	(−)-Epigallocatechin gallate	5,7,3′,4′,5′-OH, 3-gallate	Tea
Anthocyanidin	Cyanidin	3,5,7,3′,4′-OH	Cherry, strawberry
	Delphinidin	3,5,7,3′,4′,5′-OH	Dark fruits



Common Name	Abbreviation	R_1	R_2
Epicatechin	EC	H	H
Epicatechin gallate	ECG	Gallate	H
Epigallocatechin	EGC	H	Gallate
Epigallocatechin gallate	EGCG	Gallate	Gallate

FIGURE 12.3 Structures of major green tea catechins.



Common Name	Abbreviation	R_1	R_2
Theaflavin	TF	H	H
Theaflavin-3-gallate	TF3G	Gallate	H
Theaflavin-3′-gallate	TF3′G	H	Gallate
Theaflavin-3,3′-digallate	TFDG	Gallate	Gallate

FIGURE 12.4 Structures of theaflavins in black tea.

(ECG), and epicatechin (EC) (Figure 12.3). EGCG is the most abundant catechin in green, oolong, and black teas. Green and oolong teas typically contain 30–130 mg of EGCG per cup (237 mL), whereas black teas may contain up to 70 mg of EGCG per cup [55]. The main pigments in black tea are theaflavins and thearubigins, which are formed by the oxidation and polymerization of catechins during fermentation. The resulting brewed black tea contains 3–10% catechins, 2–6% theaflavins, and more than 20% thearubigins. The structures of four theaflavins are shown in Figure 12.4, on the other hand, the structures of thearubigins are not well characterized [55]. It is known that theaflavins make important contributions to the properties of black tea, such as color, taste, and mouthfeel [55].

Green tea and its constituents have been extensively studied both *in vitro* and in animal models of carcinogenesis [71]. Whereas these compounds have been shown to be efficacious in a number of animal models of carcinogenesis, the epidemiological evidence regarding the effects of tea consumption on cancer risk in humans is conflicting [71]. Numerous potential mechanisms have been proposed for the cancer-preventive activity of tea and tea constituents based on studies with cancer

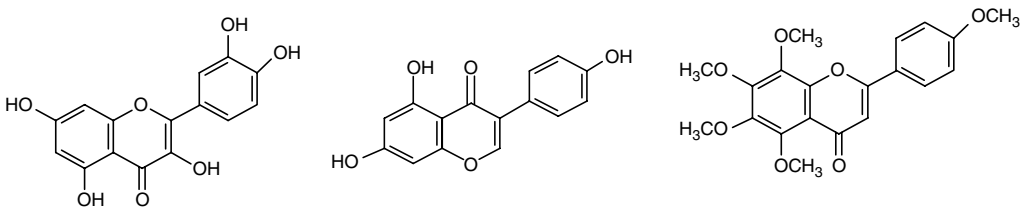


FIGURE 12.5 Structures of quercetin, genistein, and tangeretin.

cell lines. *In vitro*, tea polyphenols, especially EGCG, have been shown to cause growth inhibition and induce apoptosis in a number of human tumor cell lines including melanoma, breast cancer, lung cancer, leukemia, and colon cancer [55,71]. The relative importance of any of these mechanisms *in vivo* remains to be determined. In general, biologically important activities that can be modulated by low concentration of an agent are likely to be more relevant *in vivo*. One problem faced by most studies is the relatively high concentrations of tea compounds used in *in vitro* studies. These concentrations often far exceed those found in animal plasma or tissue following tea consumption.

Several studies of the systemic bioavailability of orally administered green tea and catechins in human volunteers have been conducted. It has been shown that oral administration of 20 mg green tea solids/kg body weight results in maximum plasma levels for EGC, EC, and EGCG of 728.8, 427.6, and 170.1 nM, respectively. Plasma EC and EGC are present mainly as the glucuronide or sulfate conjugates whereas 77% of the EGCG was in the free form [70].

Another commonly occurring flavonoid is quercetin (Figure 12.5). Quercetin is found typically in grapes, wine, tea, onions, apples, and leafy green vegetables, and has been shown to be a potent antioxidant and anti-inflammatory agent that protects the blood vessels, the cell and its structures from the harmful effects produced by free radicals. In its capacity as an antioxidant, quercetin lowers LDL cholesterol levels and provides some protection from CVDs [2]. The anti-inflammatory mechanism of action of quercetin is believed to be by the inhibition of lipoxygenase and cyclooxygenase, resulting in a reduction of the production of proinflammatory oxylipin mediators [18].

Health benefits of soybean and its products have been recognized in recent years. Genistein (Figure 12.5), which is an isoflavone, is believed to be the main nutraceutical in soybeans. As a phytoestrogen, genistein lacks estrogenic activity and exhibits antiestrogenic activity. The structures of phytoestrogens have been shown to be very similar to estrogenic steroids and this account for their antagonistic relationship. Competitive binding of phytoesterogens to cell nucleus estrogen receptors is believed to alter the production of specific proteins inside the cell; these proteins continue into the cell nucleus, bind to DNA regulatory sites, and affect protein production by increasing or decreasing gene expression [17].

Genistein has been shown, through *in vitro* studies, to inhibit the growth of various types of cancer cells, especially those that are hormone-dependent. The mechanisms of action include inhibition of many enzymes involved in tumor growth and development, and induction of apoptosis [13]. Also there is evidence of antioxidant activity, and scavenging activity of hydrogen peroxide by genistein.

Tangeretin (5,6,7,8,4′-pentamethoxyflavone) (Figure 12.5) belongs to the polymethoxylated flavones and is abundant in citrus peels [46]. Tangeretin plays an important role in every stage of cancer development. It blocks the xenobiotic cancer initiation stage by modulating hepatic Phase I and Phase II enzymes,* and it is able to block cancer promotional stages† in various ways.

* These are enzymes involved with drug and (pro)carcinogen metabolism where consecutive metabolic steps of oxygenation (Phase I) and conjugation (Phase II) usually occur.

† The promotion stage of carcinogenesis is where cancerous cells exist but a chemical signal (promoter) is required to induce neoplastic growth.

Tangeretin modulates the cell cycle* regulation pathway [46]. Compared with hydroxylated flavonoids, methoxylation increases hydrophobicity of the molecules, which facilitates cell membrane uptake and *in vivo* bioavailability. Moreover, unlike hydroxylated flavonoids, tangeretin was shown to have no genotoxicity by the Ames test over a similar range of concentrations examined [46]. On the basis of all these properties, tangeretin and other related polymethoxylated flavones seem to hold promise in dietary strategies to reduce risk of cancer and other human chronic diseases.

12.3.3 Proanthocyanidins

Proanthocyanidins are synonymous with condensed tannins and are found in fruits, berries, beans, nuts, cocoa, and wine [50]. The abundance of proanthocyanidins in plants makes them an important part of the human diet [50]. Proanthocyanidins are oligomers or polymers of flavan-3-ols and these units are linked mainly through C4→C8 bond. This linkage is called a B-type linkage. An additional ether bond between C2→C7 resulting in double linkage of the flavan-3-ol units is called an A-type [50]. Figure 12.6 shows chemical structures of the most common dimers B1, B2, and A2, as well as trimers C1 and C2.

The fact that the French have one of the lowest incidences of coronary heart disease in the Western world despite a diet with a relatively high fat content has been linked to the high consumption of red wine. This phenomenon has commonly been referred to as the "French paradox." It was suggested that the high proanthocyanidin content in red wine could explain this "French paradox." Many *in vitro* and *in vivo* studies have shown the potential cardioprotective effects of proanthocyanidins [50]. However, the data on both the bioavailability and biotransformation of proanthocyanidins in humans are limited. The available studies indicate that dietary proanthocyanidins are poorly absorbed and the majority of polymeric anthocyanidins pass unaltered through the small intestine whereafter they are degraded into small phenolic acids by the colonic microflora in large intestine [50]. However, there is evidence that proanthocyanidins from cranberry are effective in preventing urinary tract infections.

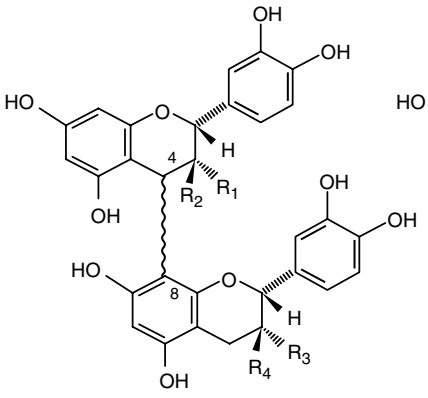
12.3.4 Other Polyphenolic Compounds

Besides flavonoids, there exist many polyphenolic compounds in food, particularly fruits, vegetables, and spices. Table 12.2 shows the health benefits of some of these compounds.

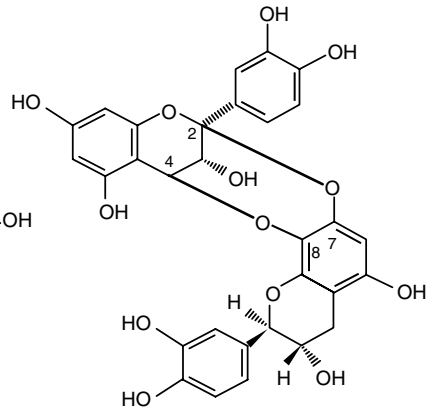
The dried rhizome of the plant *Curcuma longa* Linn. has been used for centuries as a naturally occurring medicine for the treatment of topical inflammation and other diseases. The major pigment in powdered rhizome, commonly known as turmeric spice, was identified as curcumin (Figure 12.7). The spice, turmeric, consumed daily in food in India, has been used as a food preservative and a food- and drug-coloring agent. Several laboratories have shown that curcumin and/or turmeric have potent anti-inflammatory activity [1]. Besides being a powerful antioxidant and anti-inflammatory agent, the most interesting property of curcumin is its anticarcinogenic power that relies on decreasing cell proliferation of cancer cells by inducing apoptosis. Several *in vitro* and some *in vivo* studies have demonstrated that curcumin was very effective at inducing apoptosis in several types of cancer cells [1]. Curcumin has very low bioavailability; however, some biotransformation studies on curcumin show that curcumin is present in the blood as glucuronide and glucuronide/sulfate conjugate forms [30].

Gingerols are the main pungent components of the rhizome of *Zingiber officinale*, which belongs to the ginger family *Zingiberaceae*. Common ginger has been used as a folk medicine for thousands of years. [6]-Gingerol (Figure 12.7) is the most studied gingerol in anticarcinogenic research and

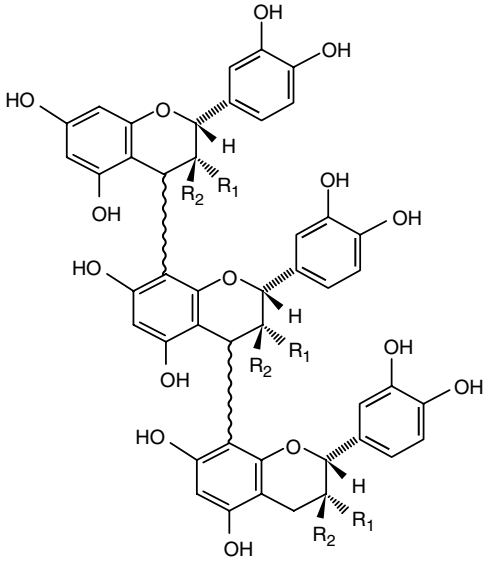
* Cell cycle is the sequential series of events required for cells to undergo division, and this cycle is regulated by specific chemicals (cyclins) and enzyme activities.



B1: R_1 = OH; R_2 = H; R_3 = H; R_4 = OH
B2: R_1 = OH; R_2 = H; R_3 = OH; R_4 = H



Dimer A_2



C1: R_1 = OH; R_2 = H
C2: R_1 = H; R_2 = OH

FIGURE 12.6 Structures of common proanthocyanidin dimers and trimers.

TABLE 12.2
Other Polyphenols, Their Dietary Source and Potential Health Benefits

Polyphenol	Dietary Source	Potential Health Benefits
Curcumin	Tumeric root	Anticarcinogenic, anti-inflammatory, cardioprotective, suppress diabetes
Gingerol	Ginger root	Anticarcinogenic, antiemetic nausea
Carnosic acid, carnosol	Rosemary, sage	Antioxidative, anticarcinogenic
Resveratrol	Grape, red wine	Anticarcinogenic, antioxidative, cardioprotective

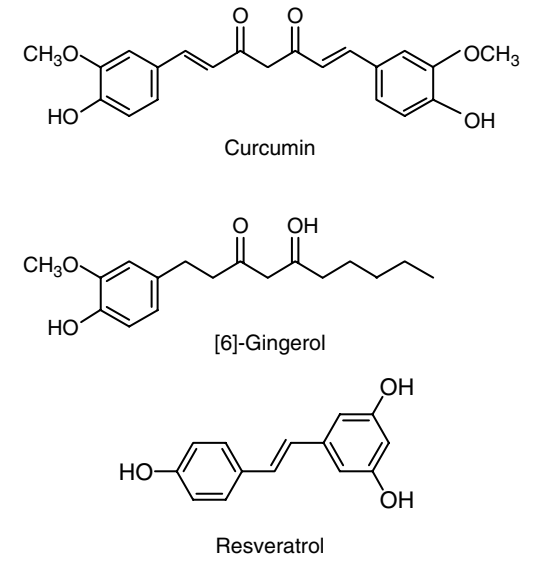


FIGURE 12.7 Structures of curcumin, [6]-gingerol, and resveratrol.

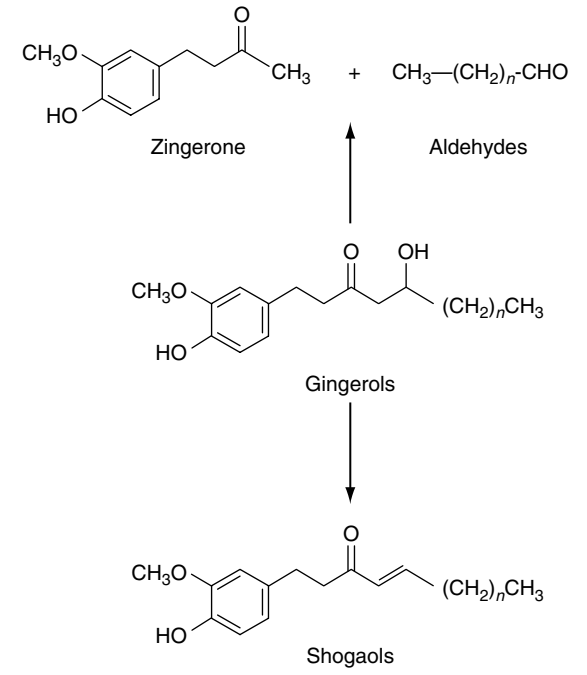


FIGURE 12.8 Degradation reaction of gingerols.

has been shown to be a potent chemopreventive agent in both *in vitro* and *in vivo* models [63]. The major metabolite of [6]-gingerol is [6]-gingerol-4′-*O*-β-glucuronide. [6]-Gingerol is cleared rapidly from the plasma with a short terminal half-life ($\sim$7 min) in rats [19].

Gingerols are sensitive to heat. During drying- or thermal-processing gingerols either dehydrate to the corresponding shogaols or are degraded by a retro-aldol reaction to zingerone and the corresponding aldehydes (Figure 12.8) [9]. The biological activity of shogaols is yet to be determined.

Resveratrol (3,5,4′-trihydroxystilbene) (Figure 12.7) is a compound found mainly in the skin of grapes, peanuts, mulberries and, most importantly, red wines. It is synthesized from *p*-coumaroyl CoA and malonyl CoA in response to stress, injury, infection, or UV-irradiation. It is classified as a phytoalexin, which confers disease resistance to plants. More recently, *in vitro* and animal experiments have shown that it exhibits many biological effects such as protection against atherosclerosis, antioxidant activity, inhibition of platelet aggregation, and antimutagenic and anticarcinogenic properties [31,69].

Rosemary and sage leaves are commonly used as spices and flavoring agents. The dried leaf of the rosemary plant is one of the most widely used spices for food processing because it has a desirable flavor and since it possesses high-antioxidant activity. Several phenolic diterpenes with antioxidant activity have been isolated from rosemary and sage leaves, the most notable one is carnosic acid. Besides antioxidant activity, both rosemary extract and carnosic acid have been shown to inhibit chemically induced tumor initiation* in animal models.

Carnosic acid is not stable during processing and storage. It will first oxidize to form carnosol, and undergo further oxidative transformation to form rosmanol. The mechanism of transformation is shown in Figure 12.9 [25]. It is interesting to note that both carnosol and rosmanol have antioxidant activity comparable to that of carnosic acid [25].

12.3.5 Sulfur-Containing Bioactives in *Allium*

Another group of phytonutrients are the sulfides, which are composed of vegetables from the *Allium* family including onions, garlic, scallions, chives, and leeks. These sulfides show many health-promoting characteristics including inhibition of cell growth and proliferation, enhancement of the immune system, alteration of carcinogen activation (by Phase I enzymes), stimulation of detoxification enzymes (Phase I and especially Phase II enzymes), and inhibition of carcinogen–DNA binding [39]. Garlic specifically has been shown to retard the progression of skin, stomach, bladder, and colon cancers, and provide protection from oxidative damage [65].

Allicin (diallyl thiosulfinate) is the major organosulfur compound identified in fresh minced garlic that contributes to the health benefits of garlic. However, allicin does not occur in intact garlic tissue. Upon cutting or crushing, alliin (*S*-allylcysteine *S*-oxide; Figure 12.10), the major compound in intact garlic cloves, is converted to allicin by the action of alliinase (illustrated in Chapters 6 and 10). Studies have shown that regular consumption of garlic can lead to a reduced risk of heart attacks and strokes, due to a decrease in the levels of total and LDL cholesterol and triglyceride, while not affecting high-density lipoprotein levels [51].

The chemical transformation of allicin upon heating yields other sulfur compounds (Figure 12.10) such as ajoenes, vinyldithiin, and allyl methyl trisulfide, which are partly responsible for increase in fribrinolytic activity and inhibition of platelet aggregation [51]. Additionally, these sulfur compounds may confer antitumor properties [51].

12.3.6 Isothiocyanates and Indoles

Compounds known as isothiocyanates and indoles are formed during the mastication of some cruciferous vegetables, which promotes thioglucosidase (myrosinase) hydrolysis of the precursor conjugates known as glucosinolates. The vegetables belonging to the *Brassica* genus, which include cabbage, broccoli, kale, turnips, cauliflower, and Brussels sprouts, are the primary source of glucosinolates and related breakdown products (see also Chapter 6). Studies have shown that

* Tumor initiation occurs when a threshold level of mutations has occurred to induce neoplasia, which is abnormal, disorganized growth.

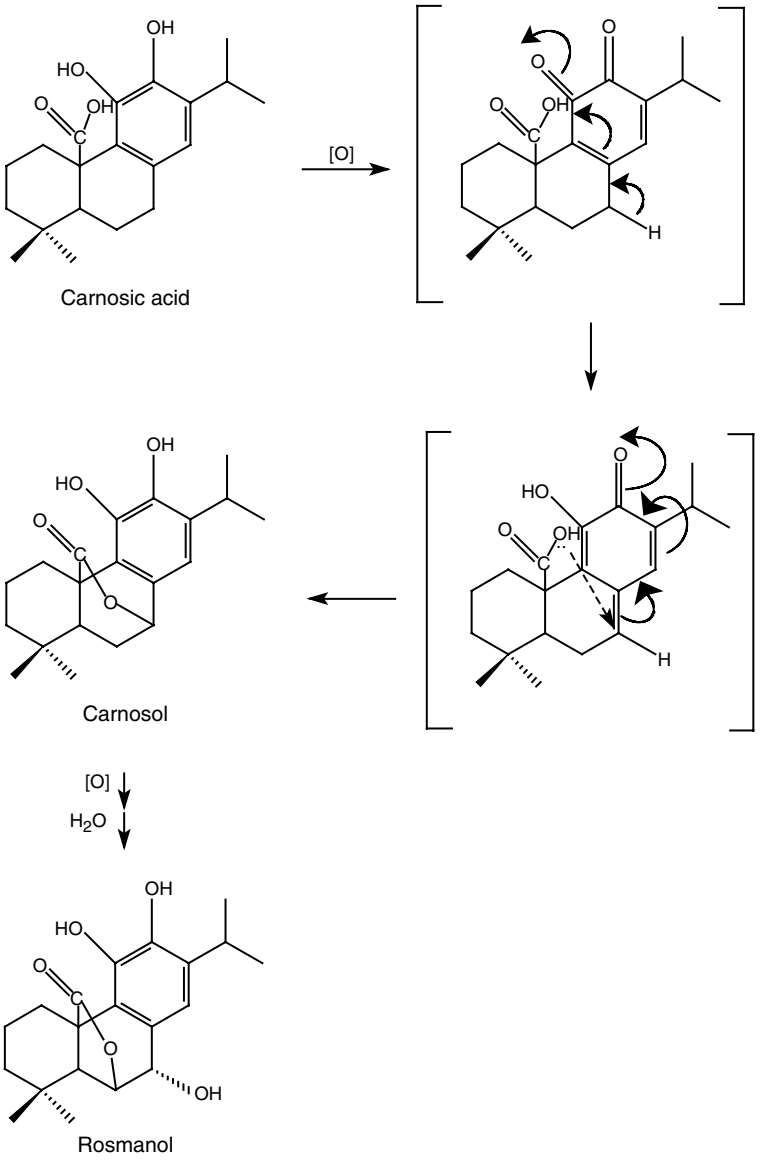


FIGURE 12.9 Oxidative transformation of carnosic acid to carnosol and then to rosmanol.

animals treated with isothiocyanates, such as phenethyl isothiocyanate, benzyl isothiocyante, and sulforaphane (4-methylsulfinylbutyl isothiocyanate) (Figure 12.11), after carcinogen exposure had reduced tumor incidence [11].

Isothiocyanates are thermally labile during processing and the major decomposition products are derivatives of thiourea. Figure 12.12 shows the decomposition pathway of sulforaphane. Sulforaphane is first hydrolyzed into an amine and the resulting amine reacts with sulforaphane to form *N*,*N*′-di(methylsulfinyl)butyl thiourea [33]. The consequence of this decomposition on bioactivity is not known.

Indole-3-carbinol is a bioactive compound that is derived from maceration-induced myrosinase hydrolysis of glucobrassicin found in cruciferous vegetables (Figure 12.13). Indole-3-carbinol has been shown to have anticarcinogenic and cancer-preventive properties, due to its ability to induce

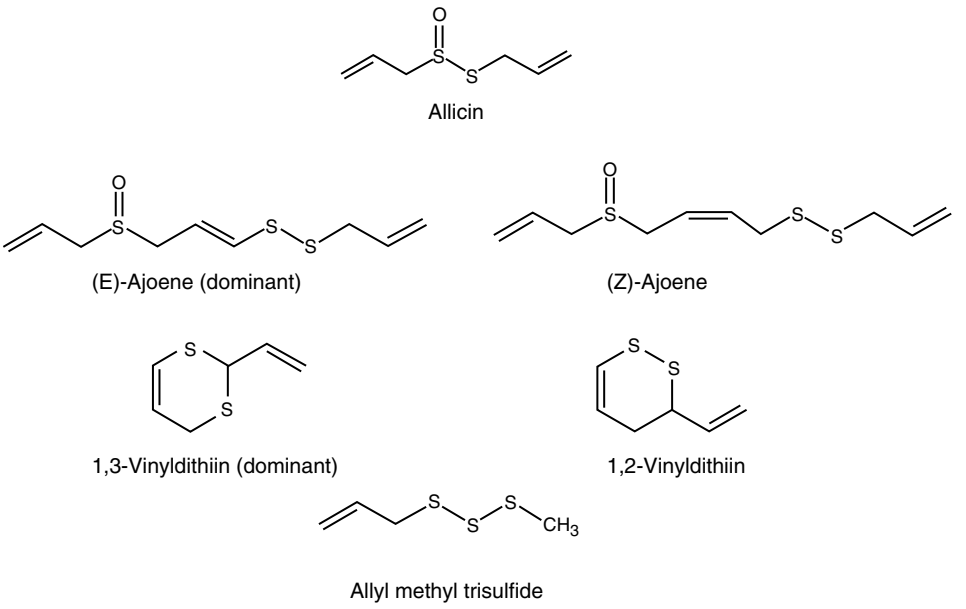


FIGURE 12.10 Structures of allicin, ajoenes, vinyldithiin, and allyl methyl trisulfide.

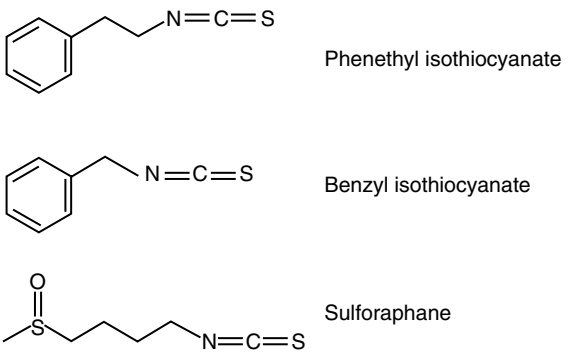


FIGURE 12.11 Structures of phenethyl isothiocyanate, benzyl isothiocyanate, and sulforaphane.

cytochrome P450 (Phase I enzyme) and glutathione *S*-transferase (Phase II enzyme) activities, thus resulting in a heightened metabolic capacity toward carcinogens [57].

12.4 GENERALIZED PROTECTIVE MECHANISMS OF PHYTOCHEMICALS

Various phytonutrients have been shown to provide a wide range of health-promoting benefits. The general protective mechanisms used by these plant-derived compounds are antioxidative protection, DNA damage protection, improved immune function, and hormone modulation [8,20,56].

Antioxidant protection from damage due to free radicals is vital for the integrity of cellular structures and macromolecules [16]. As we age, the biochemical systems that utilize antioxidants for our defense and protection also decline, and can be aggravated by the presence of various oxidative stresses caused by pollution, exercise, smoke exposure, and radiation. This defense system

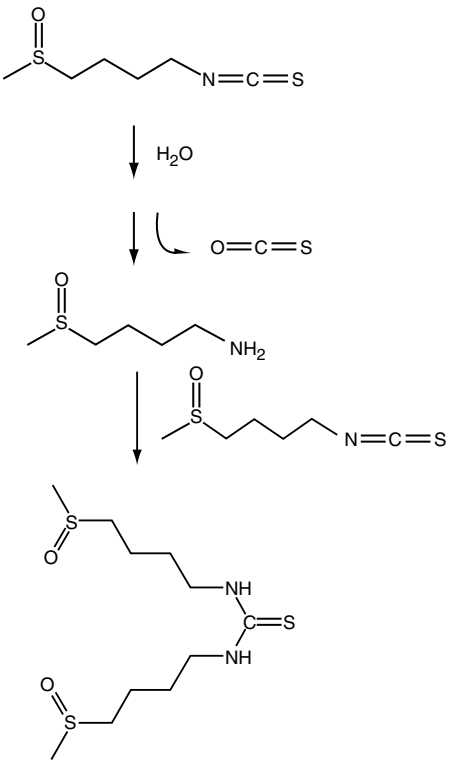


FIGURE 12.12 Pathway for the formation of *N*,*N*′-di(methylsulfinyl)butyl thiourea from sulforaphane.

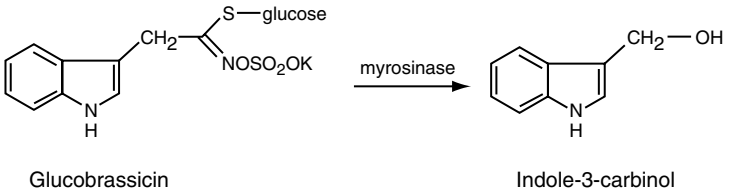


FIGURE 12.13 Formation of indole-3-carbinol from glucobrassicin by the action of myrosinase.

operates through a series of complex networks between vitamins C and E, carotenoids, zinc-, copper-, selenium-, and magnesium-dependent enzyme antioxidants, and other phytonutrients, which together perform highly integrated recycling and regeneration reactions to optimize free radical protection. Deficiencies in any of the mentioned necessary components could potentially lead to a severely compromised defense system [8,16].

Oxidative stress is created when there is an imbalance between the generation of ROS and their removal. These ROS include free radicals such as hydroxyl radical, peroxy radical, superoxide anion radical, and other reactive species such as hydrogen peroxide and singlet oxygen generated as a result of naturally occurring processes (e.g., mitochondrial electron transport, exercise), environmental stimuli (e.g., ionizing radiation from the sun), environmental pollutants, changed atmospheric conditions (e.g., hypoxia), and lifestyle stressors (e.g., cigarette smoke and excess alcohol consumption). Defense mechanisms to remove the ROS include enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and antioxidants, which can exist endogenously in the body or

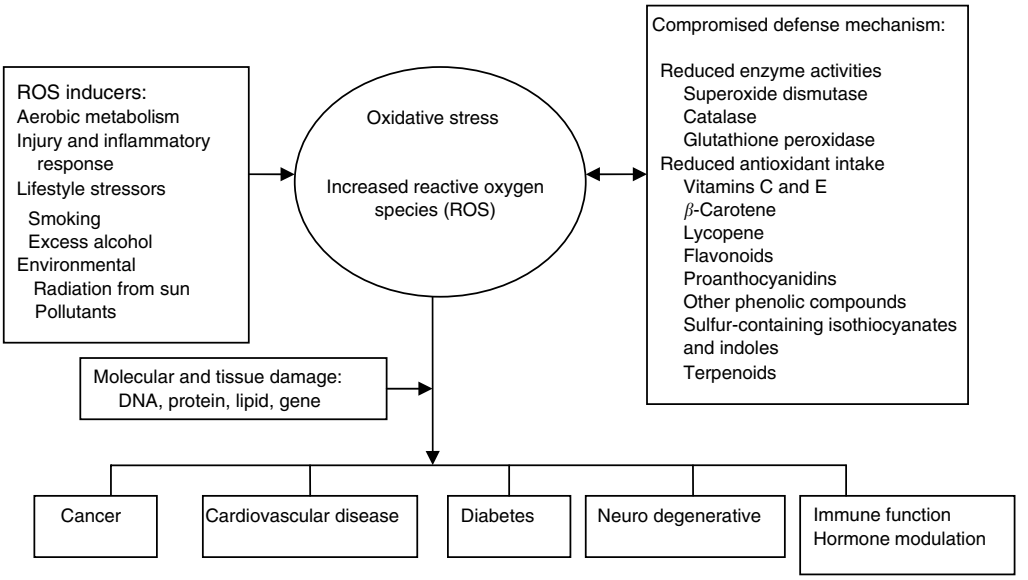


FIGURE 12.14 Oxidative stress and its possible impact.

consumed from diet. Oxidative damage can occur to macromolecules such as protein, DNA, and lipid (Figure 12.14).

DNA base alterations, strand breakage, and mutations are problems that are usually associated with free radical attacks on DNA. In studies, it has been shown that this damage can be stopped, reduced, and even reversed with antioxidant supplementation [72]. These results were seen not only in populations with high oxidative stress (smokers) but also in populations where oxidative stress was not the highest risk factor [72].

The human immune system is vital for the protection against various illness-causing agents including malignant and mutant cells. The immune system, with its bioactive compounds produced by highly interactive cells, is very susceptible to effects that can be debilitating to the system such as stress, environmental exposure, deficiencies in nutrition, and aging. Studies have shown that the use of some antioxidants improves immune function by helping to increase the number and activity of immune system cells, like natural killer cells, T-helper cells, and transferring receptor interleukin-2 dependent cells. Curcumin has been shown to have immunomodulatory characteristics and to reverse multiple sclerosis in animal models [5].

The anti-inflammatory effect of many phytonutrients has been well studied [29]. The general mode of action used to carry out this beneficial activity has been shown by the inhibition of the inducible form of cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) [29]. Both COX-2 and iNOS have been shown to be involved in antigen-stimulated inflammatory responses. Effective phytonutrients work by inhibiting messenger RNA and protein synthesis associated with COX-2 and iNOS. The anti-inflammatory mechanism is mediated through inhibiting proinflammatory cytokines* or inhibition of signaling pathways and transcription factor nuclear factor-κB† [29]. A final health-promoting mechanism demonstrated by many phytonutrients is the modulation of hormones [4]. Phytoestrogens have been shown to have cancer-retardant properties, a positive bone density impact, improved plasma lipid profile, lowered elevated serum cholesterol, and ameliorative effect on menopausal symptoms [4]. The cancer-preventive activity

* Cytokines are proteinaceous substances critical to functioning of innate and adaptive immune responses.

† Lack of regulation of this transcription factor is often associated with inflammatory responses.

is believed to be involved in part with an inhibition of angiogenesis* and/or tyrosine kinase† activity [66].

12.5 BOTANICAL DIETARY SUPPLEMENTS

With the increasing demand for the prevention of diseases, herbal products have become popular in the international market, not to mention in those countries with a long history of using herbal medicines such as China, India, Japan, and Germany. Currently there are about 750,000 plant species, 300,000 registered species, 30,000–75,000 medicinal plants, 20,000 medicinal plants listed by World Health Organization (WHO), and 400 medicinal plants widely traded in the world.

In the United States, herbal products are mainly regulated as dietary supplements and sold in different forms such as fresh plant products, dried botanical powders, liquid botanical extracts, soft extracts, dry extracts, tinctures, and purified natural compounds. The open market of the world allows all kinds of botanical products to be sold in the United States with ginseng, ginkgo, garlic, saw palmetto, echinacea, soy, bilberry, grape seed, and green tea extracts among the top-selling ones. Table 12.3 lists some of the popular botanical dietary supplements, and the presumptive active components and functions [12].

For different commercial purposes, there are different quality control and quality assurance standards for these botanical dietary supplements. But usually they follow some general rules; the botanicals must be authenticated, safe to use, with a limit of foreign materials, heavy metals, aflatoxins, and pesticides. The pH value, ash contents, moisture contents, and particle size should be in a reasonable range. Also, microbiological tests must be passed. The general quality control standards are usually able to be met, but specific standards for herbal products are usually lacking [67].

12.6 PROCESS-INDUCED NUTRACEUTICALS IN FOODS

Almost all food items consumed are processed to some extent. Processing of food plays a crucial role in food quality, safety, storage, and characteristics of the specific food item. The chemical reactions involved in food processing are numerous and complicated. Although some bioactive compounds are destroyed in the process, many more initially not present in intact tissue or raw material are generated. Some of these compounds have a great potential to promote good health.

Hydroxycinnamic acids such as ferulic acid and caffeic acid are widely distributed in nature. They exist in a free form or as simple esters in fruits, vegetables, and cereals. The concentration of hydroxycinnamic acid related compounds in coffee beans is high. Green coffee beans contain ~6–9% chlorogenic acid, which is the quinic acid ester of caffeic acid [14]. During the roasting process, the chlorogenic acid content decreases dramatically. Roasted coffee shows anticarcinogenic and antibacterial activities, suggesting that the degradation products of chlorogenic acid have some bioactivity [15].

Caffeic acid is one of the major catecholic constituents in green coffee. Under thermal conditions, caffeic acid undergoes rapid decarboxylation to form simple catechol monomers as well as more complex condensed cyclic dimers and polymers. Two major compounds, 1,3-*cis*- and 1,3-*trans*-tetraoxygenated phenylindans, were isolated as mild pyrolysis products of caffeic acid. *In vitro* studies indicate that these two compounds have potent antioxidant and antimutagenic activities. Figure 12.15 shows the mechanism for their formation. These two compounds were found to be present at levels ranging from 10 to 15 ppm in roasted coffee [58].

* Growth of new blood vessels, a process especially required by fast-growing cancer cells.
† An enzyme involved in signal transduction, especially as related to cell proliferation.

TABLE 12.3
Popular Botanical Dietary Supplements, Their Potential Active Components and Functions

Dietary Supplements	Latin Name	Potential Active Components	Major Potential Health Benefit
Astragalus	*Astragalus membranaceus*	Polysaccharides, saponins (astragalosides)	Immunomodulatory, hepatoprotective
Black cohosh	*Cimicifuga racemosa*	Fukinolic acid 23-epi-26-deoxyactein	Relief of menopausal symptoms
Cranberry	*Vaccinium macrocarpon*	Proanthocyanidins	Prevention and treatment of urinary tract infections
Dang Gui	*Angelica sinensis*	Ligustilide	Treatment of gynecological conditions
Echinacea	*Echinacea purpurea*, *E. pallida*, *E. angustifolia*	Polysaccharides and glycoproteins, cichoric acid, alkamides	Treatment of common cold, cough, and upper respiratory infections
Feverfew	*Tanacetum parthenium*	Parthenolide and other sesquiterpene lactones	Alleviation of fever, headache, and women's ailments
Garlic	*Allium sativum*	Allyl sulfur compounds	Antibacterial, anticarcinogenic, antithrombotic, hypolipidemic
Ginger	*Zingiber officinale* Roscoe	Gingerols	Antiemetic, anti-inflammatory, digestive aid
Ginkgo biloba	*Ginkgo biloba*	Ginkgolids, flavonoids	Treatment of cerebral dysfunction and circulatory disorders
American ginseng	*Panax quinquefolium*	Ginsenosides	Therapeutic effects on immune function, cardiovascular diseases, cancer, sexual function
Asian ginseng	*Panax ginseng*	Ginsenosides	Combat psychophysical tiredness and asthenia
Goldenseal	*Hydrastis canadensis*	Alakaloid berberine and β-hydrastine	Soothing irritated skin and mucous membranes, easing dyspepsia
Grape seed extract	*Vitis vinifera*	Proanthocyanidins	Antioxidant, anti-inflammatory, immunostimulatory, antiviral, and anticancer
Green tea polyphenols	*Camellia sinensis*	Epigallocatechin gallate and catechins	Preventive effects on heart diseases, cancer, neurodegenerative disorders, and diabetes
Kava	*Piper methysticum*	Kava lactones	Effects on relaxing and mood calming
Licorice	*Glycyrrhiza glabra*	Triterpene saponins, flavonoids and other phenolics	Possess soothing, anti-inflammatory, and antitussive properties
Maca	*Lepidium meyenii*	Aromatic isothiocyanates	Use for aphrodisiac purpose
Milk thistle	*Silybum marianum*	Silymarin	Treatment of liver disorders
Pycnogenol	*Pinus pinaster* ssp. *Atlantica*	Procyanidins	Use for protection of circulation, and to restore capillary healing
Red clover	*Trifolium pratense*	Isoflavones	Treatment for menopausal symptoms
Reishi mushroom	*Ganoderma lucidum*	Triterpenoids, polysaccharides	Antitumor and immunomodulating effects
Saw Palmetto	*Serenoa repens*	Unknown	Use for prostate health
Soy isoflavones	*Glycine max*	Genistein, daidzein	Prevention of menopausal symptoms, osteoporosis, coronary heart disease, and cancer
St John's wort	*Hypericum perforatum*	Hyperforin, hypercin	Treatment of mild depression
Valerian	*Valeriana officinalis* L.	Valepotriates (iridoids)	Use for mild sedative and sleep disturbance
Yohimbe	*Pausinystalia johimbe*	Yohimbine	Use for aphrodisiac purpose

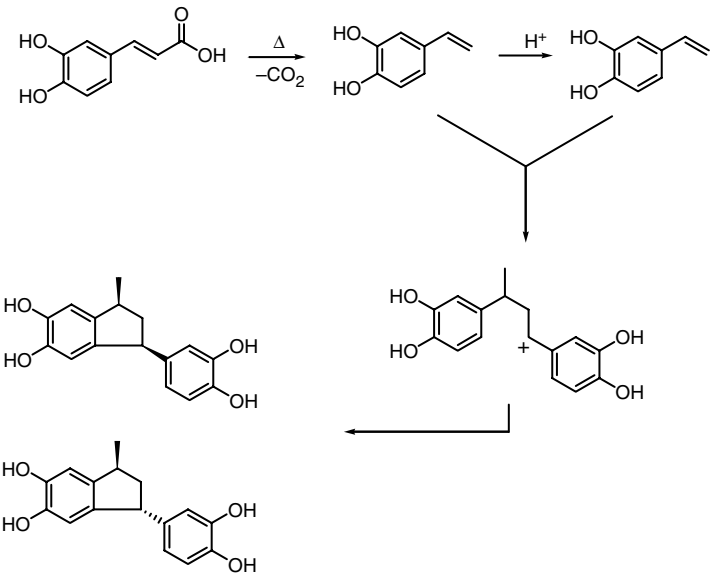


FIGURE 12.15 Formation of 1,3-tetraoxygenated phenylindans from caffeic acid.

Thermal energy is not the only factor that will transform bioactive agents in food. Acid treatment during the processing of certain food may also cause the conversion of phytochemicals. Formation of active antioxidant in sesame oil during the refining process is a good example. Sesamolin is the major lignan in sesame seeds. During the refining process, the concentration of sesaminol increased to 0.5–1.0 mg/g oil as a result of high yield conversion from sesamolin to sesaminol via intramolecular group transfer catalyzed by acid clay used for bleaching [22]. Sesaminol is responsible for the antioxidant activity of unroasted sesame oil. Figure 12.16 shows the formation mechanism of sesaminol. Many lignans are considered phytoestrogens, as are isoflavones.

12.7 BIOAVAILABILITY OF BIOACTIVE COMPOUNDS

Bioavailability is the degree to which a drug, nutrient, dietary supplement, or nutraceutical is available to the body. Bioavailability is influenced by how much of the substance is absorbed and circulated in the human body. Problems with bioavailability arise when trying to elucidate exactly what dose brings about the desired physiological response. Manufacturers producing 500 mg vitamin C pills cannot claim that 500 mg of the vitamin are taken in and used by the body.

There is variance between different human subjects and their uptake of certain food-based chemicals. This means that two people taking the same dose may absorb different amounts of the same compound. This disparity is due to variability in absorption, distribution, metabolism, and excretion (abbreviated as ADME) of the pharmaceutical or dietary agent. Because of this one individual may get less benefit of a prescribed dose than another, and taking a 500 mg pill does not guarantee that a body has the ability to utilize all 500 mg. In addition, it is sometimes the case that the ingested chemical is not the final bioactive agent. Many molecules enter the digestive system in one form only to be transformed into other metabolites that interact through absorption. The metabolic fates of many ingested bioactive agents remain to be identified to sufficiently understand the nature or extent of their bioavailability. For example, studies have shown that 11% of caffeic acid and trace amounts of chlorogenic acid, present in coffee, are found in urine [42]. The exact fate of the remaining caffeic acid is unknown. This type of ambiguity poses difficulties in understanding bioavailability and efficacy and this dilemma applies to most nutraceutical agents.

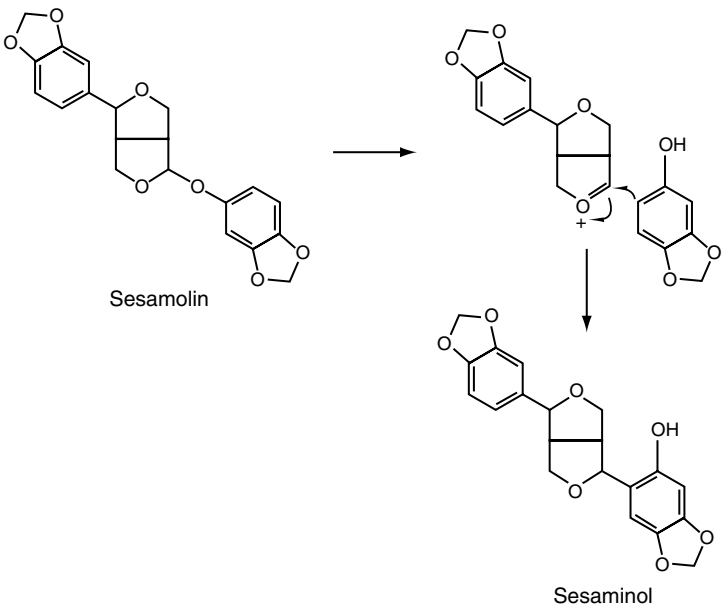


FIGURE 12.16 Formation of sesaminol from sesamolin.

Although it is difficult to say for sure how much of a compound will be taken in and used by an organism, there is some strong evidence showing how low some of the uptakes can be. In a study using chlorogenic acid, only 1.7% was recovered unchanged in the urine. In these cases, the colon could play a larger role in the metabolism of polyphenols [43].

Polyphenols reaching the colon can be broken into smaller metabolites by colonic microbiota found in the colon [43]. These bacteria are able to break down phenols, allowing absorption of these smaller metabolites by the kidneys, liver, and other organs. Later, these smaller metabolites find their way into the urine. Without a clear understanding of the chemical nature of these metabolites, we cannot screen for them in the urine. The same study also concluded that a large part of the ingested polyphenols will probably never enter the peripheral circulation as smaller metabolites [43].

The problem of reduced antioxidant activity found in smaller metabolites of larger parent compounds increases the uncertainty of bioavailability studies. An organism is not likely to absorb an entire dose, and it is likely that the compound will be broken down into smaller, unidentified compounds. Further studies are warranted to identify these compounds. Considering these variables, it is very difficult to predict the total effect of nutraceuticals, dietary supplements, or phytochemicals on host cells.

12.8 NATURAL PLANT TOXICANTS

There is a wide variety of naturally occurring plant-based (and thus food-based) toxicants and antinutrients that have teleological origins in the protection of the host plant against predation (Table 12.4, [48]) [3]. Some of these toxins are considered carcinogens (Table 12.5) and some fat-soluble ones can even bioaccumulate [49]. However, only a few such inherent toxicants have actually caused harm to persons consuming normal diets.

There is one type of toxicity called pyrrolizidine alkaloid poisoning caused by plant material that contains these specific alkaloids [52]. These alkaloids are widely distributed in the plant kingdom and are found in a large number of genera, including *Boragenaceae*, *Compositae*, and *Legummosae*, to name a few. The basic ring structure is shown in Figure 12.17. Exposure can occur in a variety of

TABLE 12.4
Examples of Inherent Toxicants in Plants

Toxins	Chemical Nature	Main Food Source	Major Toxicity Symptoms
Protease inhibitor	Proteins (molecular weight 4,000–24,000)	Beans (soy, Mung, kidney, navy, lima); chick-pea; peas; potatoes (sweet, white); cereals	Impaired growth and food utilization; pancreatic hypertrophy
Hemagglutinins	Proteins (molecular weight 10,000–124,000)	Beans (castor, soy, kidney, black, yellow, jack); lentils; peas	Impaired growth and food utilization; agglutination of erythrocytes *in vitro*; mitogenic activity to cell cultures *in vitro*
Saponins	Steroid or triterpene glycosides	Soybeans, sugar beets, peanuts, spinach, asparagus	Hemolysis of erythrocytes *in vitro*
Glucosinolates (specific ones)	Thioglycosides	Cabbage and related species; turnips, rutabaga; radish; rapeseed; mustard	Hypothyroidism and thyroid enlargement
Cyanogens	Cyanogenic glucosides	Peas and beans; pulses; linseed; flax; fruit kernels; cassava	HCN poisoning
Gossypol pigments	Gossypol	Cottonseed	Liver damage; hemorrhage; edema
Lathyrogens	β-Aminopropionitrile and derivatives	Chick-pea; vetch	Neurolathyrism (central nervous system damage)
Allergens	Proteins?	Practically all foods (particularly grains, legumes, and nuts)	Allergic responses in sensitive individuals
Cycasin	Methylazoxy-methanol	Nuts of *Cycas* genus	Cancer of liver and other organs
Favism	Vicine and convicine (pyrimidine β-glucosides)	Fava beans	Acute hemolytic anemia
Phytoalexins	Simple furans (ipomeamarone)	Sweet potatoes	Pulmonary edema; liver and kidney damage
	Benzofurans (psoralens)	Celery; parsnips	Skin photosensitivity
	Acetylenic furans (wyerone)	Broad beans	Cell lysis *in vitro*
	Isoflavonoids (pisain and phaseolin)	Peas, French beans	
Pyrrolizidine alkaloids	Dihydropyrroles	Families Compositae and Boraginaccael herbal teas	Liver and lung damage; carcinogens
Safrole	Allyl-substituted benzene	Sassafras; black pepper	Carcinogens
α-Amantin	Bicyclic octapeptides	*Amanita phalloides*; mushrooms	Salivation; vomiting; convulsions; death
Atractyloside	Steroidal glycoside	Thistle (*Atractylis gummifera*)	Depletion of glycogen

Source: Pariza, M. W. (1996). Toxic substance. In *Food Chemistry*, 3rd ed. (O. R. Fennema, ed.), Marcel Dekker, Inc., New York, NY, pp. 825–840.

TABLE 12.5
Some Naturally Occurring Carcinogens Inherent in Food

Rodent Carcinogen	Plant Food	Concentration (ppm)
5-/8-Methoxypsoralen	Parsley	14
	Parsnip, cooked	32
	Celery	0.8
	Celery, new cultivar	6.2
	Celery, stressed	25
p-Hydrazinobenzoate	Mushrooms	11
Glutamyl *p*-hydrazinobenzoate	Mushrooms	42
Sinigrin (allyl isothiocyanate)	Cabbage	35–590
	Collard greens	250–788
	Cauliflower	12–66
	Brussels sprouts	110–1,560
	Mustard (brown)	16,000–72,000
	Horseradish	4,500
Estragole	Basil	3,800
	Fennel	3,000
Safrole	Nutmeg	3,000
	Mace	10,000
	Pepper, black	100
Ethyl acrylate	Pineapple	0.07
Sesamol	Sesame seeds (heated oil)	75
α-Methylbenzyl alcohol	Cocoa	1.3
Benzyl acetate	Basil	82
	Jasmine tea	230
	Honey	15
	Coffee (roasted beans)	100
Caffeic acid	Apple, carrots, celery, cherry, eggplant, endive, grapes, lettuce, pear, plum, and potato	50
	Absinthe, anise, basil, caraway, dill, marjoram, rosemary, sage, savory, tarragon, and thyme	>1,000
	Coffee (roasted beans)	1,800
	Apricot, cherry, peach, and plum	50–500
Chlorogenic acid (caffeic acid)	Coffee (roasted beans)	21,600
Neochlorogenic acid (caffeic acid)	Apple, apricot, broccoli, brussels sprout, cabbage, cherry, kale, peach, pear, and plum	50–500
	Coffee (roasted beans)	11,600

Source: Adapted from Pariza, M. W. (1996). In *Food Chemistry*, 3rd edn. (O. R. Fennema, ed.), Marcel Dekker, Inc., New York, NY, pp. 825–840.

ways from the milk of a cow that has consumed an alkaloid-containing forage, to the consumption of a processed grain that was cultivated and subsequently processed with a weed containing these alkaloids [52]. This toxicant can cause moderate to severe liver damage, gastrointestinal problems like vomiting, abdominal pain, and ascites formation, and can result in death.

There are also some toxins that are found at fairly low levels in plants that are consumed on a daily basis all over the world. The toxin family cyanogenic glycosides are a concern, because once ingested they are metabolized to HCN [52]. Three cyanogenic glycosides have been identified in edible plants: amygdalin (benzaldehyde cyanohydrin β-glucosido-6-β-glucoside), dhurrin (*p*-hydroxybenzaldehyde cyanohydrin glucoside), and linamarin (acetone cyanohydrin glucoside)

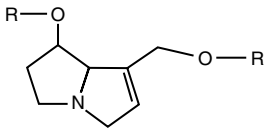


FIGURE 12.17 General structure of pyrrolizidine alkaloids.

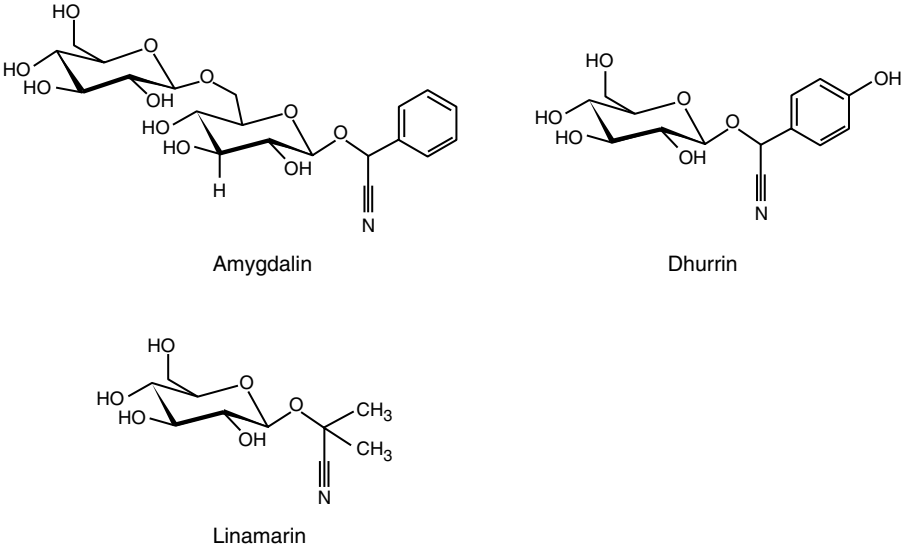


FIGURE 12.18 Structures of amygdalin, dhurrin, and linamarin.

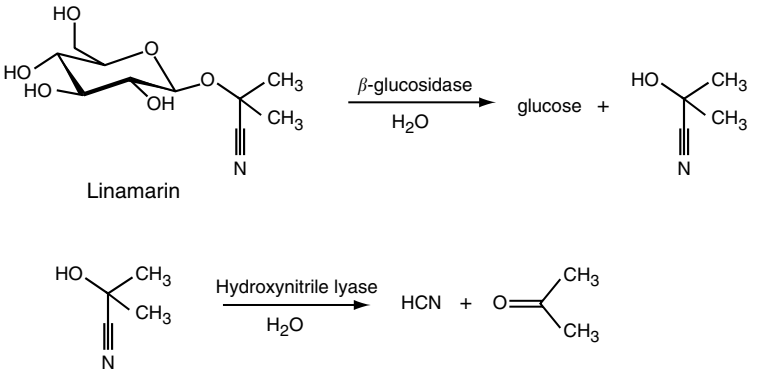


FIGURE 12.19 Release of HCN from hydrolysis of linamarin.

[52] (Figure 12.18). Amygdalin is present in bitter almonds, dhurrin in sorghum, and linamarin in pulse, linseed, and cassava. It has been reported that apricot seeds contain 2.92 mg HCN/g (degradation product of a cyanogenic glucoside) and peach seeds contain 2.69 mg HCN/g, while apple seeds contain only 0.61 mg HCN/g [26]. Figure 12.19 shows the mechanism for the release of HCN from hydrolysis of linamarin.

Two glycosides of alkaloids, α-solanine and α-chaconine (Figure 12.20), present in potatoes are toxic at higher concentration. The normal content of total glycoalkaloids in potato ranges

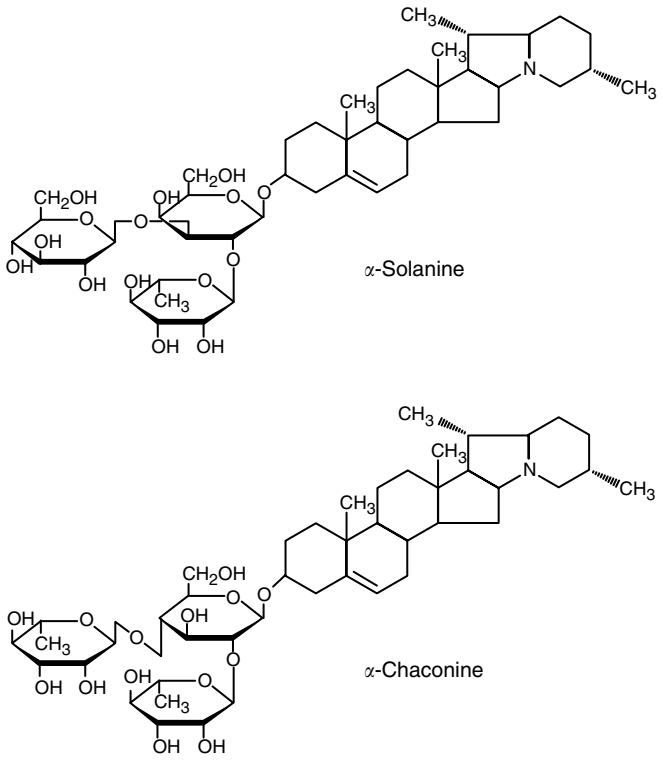


FIGURE 12.20 Structures of α-solanine and α-chaconine.

between 2 and 10 mg/100 g. FDA regulations limit the solanine content in potatoes to not more than 20 mg/100 g [52].

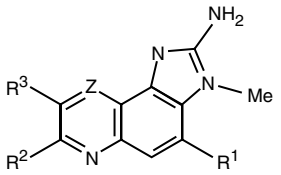
Other toxicants such as gossypol and several closely related pigments are in pigment glands of cottonseed. They can cause a variety of toxic symptoms in domestic animals (reduced fertility and growth rate). They also cause a reduction in the nutritive value of cottonseed flour. Oxalates are antinutritional factors found in spinach, rhubarb, and tomatoes that can reduce the solubility of zinc, iron, and calcium [52].

12.9 PROCESS-INDUCED TOXICANTS IN FOODS

The chemical reactions initiated in food by heat can be beneficial, as in the case of the formation of desirable flavors, or harmful, as in the generation of toxic chemicals. Two of these toxic chemicals are heterocyclic aromatic amines and acrylamide.

Heating proteinaceous food products, such as meat, may induce the formation of traces of heterocyclic aromatic amines, particularly, aminoimidazoazaarenes (AIA), which are potent mutagens [62]. Figure 12.21 shows commonly occurring AIAs. 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) have been isolated from boiled, sun-dried sardines [36]. 2-Amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), IQ, and MeIQ have been isolated from fried meat products [32].

Although the exact mechanism for the formation of heterocyclic aromatic amines in food has not been elucidated, it has been suggested that the 2-aminoimidazo part of the molecules originates from naturally occurring creatine in muscle [32]. The quinoline and quinoxaline parts are believed



Abbreviation	Z	R^1	R^2	R^3
IQ	C	H	H	H
MeIQ	C	Me	H	H
MeIQx	N	H	H	Me
4,8-DiMeIQx	N	Me	H	Me

FIGURE 12.21 Structures of commonly occurred aminoimidazoazaarenes (AIAs).

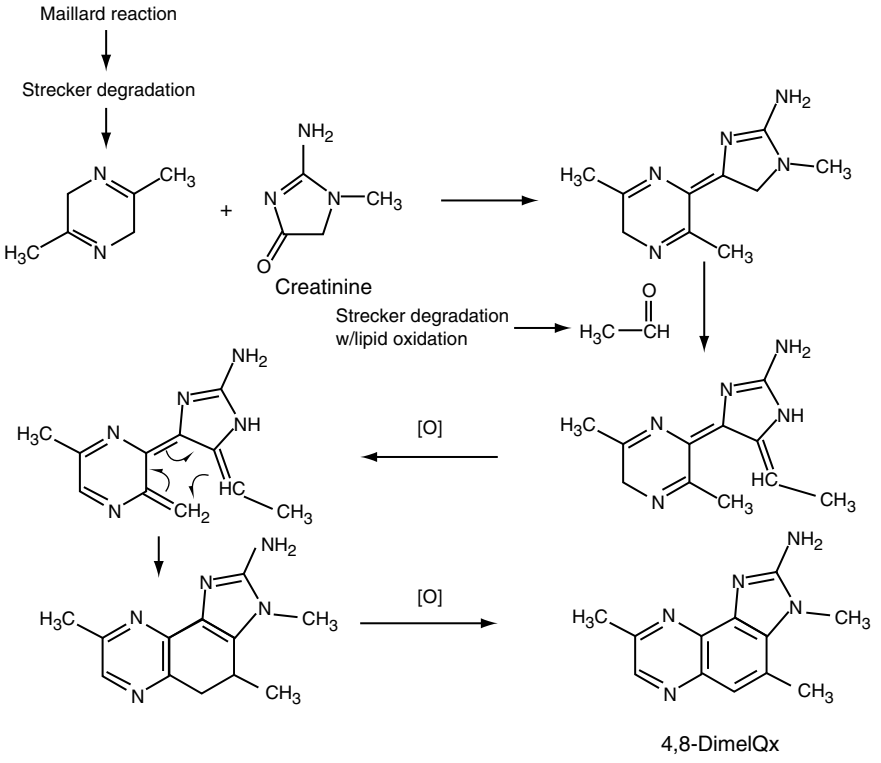


FIGURE 12.22 Mechanism for the formation of 4,8-DiMeIQx.

to be formed from Maillard reaction products, especially precursors of pyrazines or pyridines and aldehydes [32]. Figure 12.22 shows a probable mechanism for the formation of 4,8-DiMeIQx. The first step of the mechanism is the Maillard reaction between the reducing sugar and amino acids to form reactive dicarbonyl compounds such as pyruvaldehyde. Then the Strecker degradation between a dicarbonyl compound and amino acid will generate a reactive dihydropyrazine molecule. Finally, the condensation among dihydropyrazine, creatinine, and acetaldehyde will lead to 4,8-DiMeIQx. Acetaldehyde is the Strecker degradation product of amino acids such as alanine and cysteine. On the other hand, acetaldehyde is also an important product of lipid oxidation. This may be the reason why the presence of triacylglycerols facilitates the formation of heterocyclic aromatic amines [34].

Another potential dietary toxicant generated by the Maillard reaction is acrylamide. Acrylamide is a well-studied neurotoxin. It also exhibits reproductive toxicity, genotoxicity, and carcinogenicity in animals [21]. In 2002, it was reported that high amounts of acrylamide can be found in carbohydrate-rich foods subjected to thermal processing [64]. Several publications have shown that the Maillard reaction is the major reaction pathway for the formation of acrylamide [40,59]. The mechanism

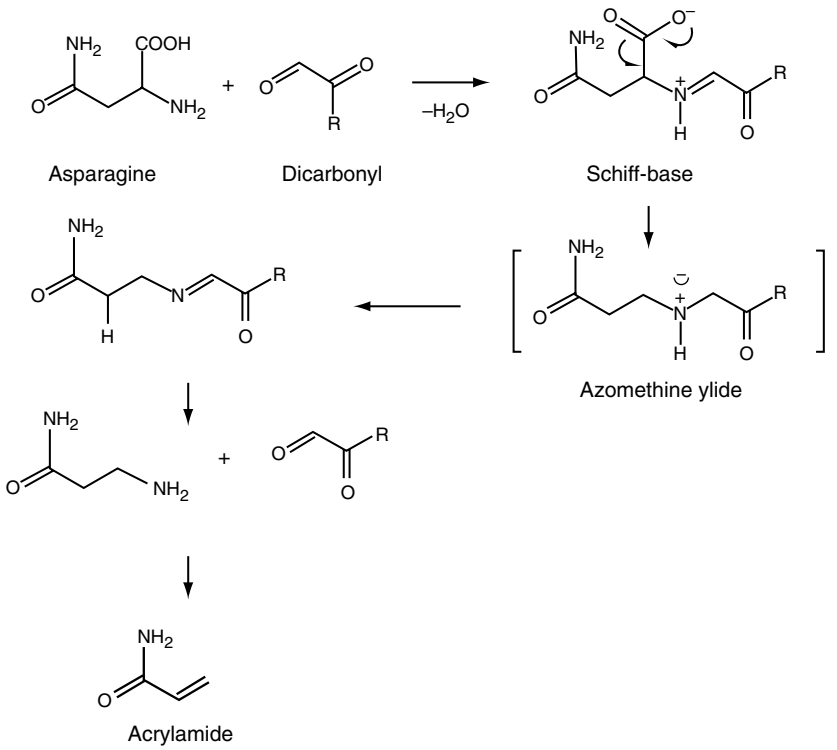


FIGURE 12.23 Mechanism for the formation of acrylamide from asparagine through the early Maillard reaction.

for the formation of acrylamide from asparagine through the early Maillard reaction is shown in Figure 12.23 [59]. There is a great concern about the possible health risks to humans due to dietary acrylamide; however, a population-based study found no association between dietary acrylamide and cancer of the large bowel, kidney, and bladder [41].

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Part III

Food Systems

13 Dispersed Systems: Basic Considerations

Pieter Walstra and Ton van Vliet

CONTENTS

13.1 INTRODUCTION

The subjects discussed in this chapter are rather different from most of the material in this book, in the sense that true chemistry, which concerns reactions involving electron transfer, is hardly involved. Nevertheless, many aspects of dispersed systems are important to an understanding of the properties of most foods and the manufacture of "fabricated foods."

Although the treatment involves some basic theory, we have tried to keep this at a minimum. Most topics discussed in this chapter are discussed in more detail in the senior author's textbook on *Physical Chemistry of Foods* (see Further Readings).

13.1.1 Foods as Dispersed Systems

Most foods are dispersed systems. A few are homogeneous solutions, such as cooking oil and some drinks, but even beer—as consumed—has a foam layer. The properties of a dispersed system cannot be fully derived from its chemical composition, since they also depend on physical structure. The structure can be very intricate as is the case with foods derived from animal or vegetable tissues; these are discussed in Chapters 16 and 17. Manufactured foods, as well as some natural foods, may have a somewhat simpler structure: beer foam is a solution containing gas bubbles; milk is a solution containing fat droplets and protein aggregates (casein micelles); plastic fats consist of oil and aggregated triacylglycerol crystals; a salad dressing may be just an emulsion; and several gels consist of a network of polysaccharide molecules that immobilize a solution. But other manufactured foods are structurally complicated in that they contain several different structural elements of widely varying size and state of aggregation: filled gels, gelled foams, materials obtained by extrusion or spinning, powders, margarine, dough, bread, and so forth.

The existence of a dispersed state has some important consequences:

1. Since different components are in different compartments, there is no thermodynamic equilibrium. To be sure, even a homogeneous food may not be in equilibrium, but for dispersed

systems this is a much more important aspect. It may have significant consequences for chemical reactions, as is briefly discussed in Section 13.1.3.

2. Flavor components may be in separate compartments, which will slow down their release during eating. Moreover, compartmentalization of flavor components may lead to fluctuations in flavor release during eating, thereby enhancing flavor, because it offsets to some extent adaptation of the senses to flavor components. Most "compartmentalized" foods taste quite different from the same food that has been homogenized before eating.
3. If, as is often the case, attractive forces act between structural elements, the system has a certain consistency, which is defined as its resistance against permanent deformation. This may be an important functional property as it is related to attributes such as stand-up, spreadability, or ease of cutting. Moreover consistency affects mouthfeel, as does any physical inhomogeneity of the food; food scientists often lump these properties under the word texture.
4. If the product has a significant consistency, any solvent present—in most foods water—will be immobilized against bulk flow. Transport of mass (and generally of heat also) then has to occur by diffusion rather than convection. This may have a considerable effect on reaction rates.
5. The visual appearance of the system may be greatly affected. This is due to the scattering of light by structural elements, provided they are larger than about 50 nm. Large inhomogeneities are visible as such and give rise to what is the dictionary meaning of texture.
6. Since the system is physically inhomogeneous at a microscopic scale, it may be physically unstable. Several kinds of change can occur during storage, which may be perceived as the development of macroscopic inhomogeneity, such as separation into layers. Moreover, during processing or usage, changes in the dispersed state may occur, which may be desirable—as in the whipping of cream; or undesirable—as in overwhipping of cream, where butter granules are formed.

In this chapter, some of the above aspects will be discussed. Large-scale mechanical properties will be largely left out, and so will aspects of hydrodynamics and process engineering. Of course, most foods show highly specific behavior, but treating them all would take very much space and provide little understanding. Therefore, some general aspects of fairly simple model dispersions will be emphasized.

13.1.2 Characterization of Dispersions

A *dispersion* is a system of discrete particles in a continuous liquid. When the particles are gaseous, we speak of a foam, with liquid particles we have an emulsion, and with solid particles a suspension (e.g., orange juice containing cell fragments). Emulsions can be of two types, oil-in-water (o/w) and water-in-oil (w/o). Most food emulsions are of the o/w type (milk, salad dressings, most soups); they can be diluted with water. Dispersions can contain a number of different particles: milk also contains small protein aggregates, and soups tend to contain pieces of vegetable tissue. Butter and margarine contain aqueous droplets, but they are not true w/o emulsions, as the oil contains fat crystals that have formed a space-filling network.

The latter is one example of a solid dispersion, that is, a system in which the continuous mass has been given solid-like properties after the dispersion has been made. In a foam omelette, the continuous protein solution has gelled. Liquid chocolate is a dispersion of solid particles (sugar crystals, cacao bean fragments) in oil, and upon cooling the oil turns into a largely crystalline fat matrix.

If a binary system is solid-like, it can in principle have two continuous "phases." The prime example is a wet sponge, where matrix and water both are continuous. Several foods are bicontinuous

systems; for instance, in bread both the gas and the solid matrix are continuous. If not, the bread would lose most of its volume after baking: the hot gas cells would shrink considerably upon cooling, since they largely consist of water vapor.

A *colloid* is usually defined as a dispersion containing particles that are clearly larger than small molecules (say, solvent molecules), yet too small to be visible. This would imply a size range of about 10 nm to 0.1 mm. Two types of colloids are usually distinguished: lyophilic ("solvent loving") and lyophobic ("solvent hating"). The latter type consists of two (or more) phases, such as air, oil, water, or various crystalline materials. Lyophobic colloids do not form spontaneously: it costs energy to disperse the one phase into the (continuous) other phase, and the system formed is not in equilibrium, and hence physically unstable.

A lyophilic colloid forms by "dissolving" a material in a suitable solvent, and the system then is in equilibrium. The main examples are macromolecules (polysaccharides, proteins, etc.) and association colloids. The latter are formed from amphiphilic molecules, such as soaps. These have a fairly long hydrophobic "tail" and a smaller polar (i.e., hydrophilic) "head." In an aqueous environment, the molecules tend to associate in such a way that the tails are close to each other and the heads are in contact with water. In this way, micelles or liquid crystalline structures are formed. Micelles will be briefly discussed in Section 13.2.2, and liquid crystalline phases [36] are not very prominent in foods.

It may further be noted that an unstable system may appear to be stable (i.e., does not show a significant change in properties during the observation time). This means that the rate of change is very small, which is often due to (1) a high activation (free) energy for a chemical reaction or a physical change to occur; or (2) a very slow motion of molecules or particles due to extremely high viscosity of the system (as in dried foods).

The *size scale* of structural elements in foods can vary widely, spanning a range of six orders of magnitude (Figure 13.1). A water molecule has a diameter of about 0.3 nm, whereas a typical cell in plant or animal tissues will be about 0.3 mm. The shape of the particles is also important, as is their volume fraction ϕ (i.e., the proportion of the volume of the system that is taken up by the particles). All these variables affect product properties. Some effects of size or scale are as follows:

1. *Visual appearance*. An o/w emulsion, for example, will be almost transparent if the droplets have a diameter of 0.03 μm; bluish white if 0.3 μm; white if 3 μm; and the color of the oil (usually yellow) will be discernable for 30 μm droplets.

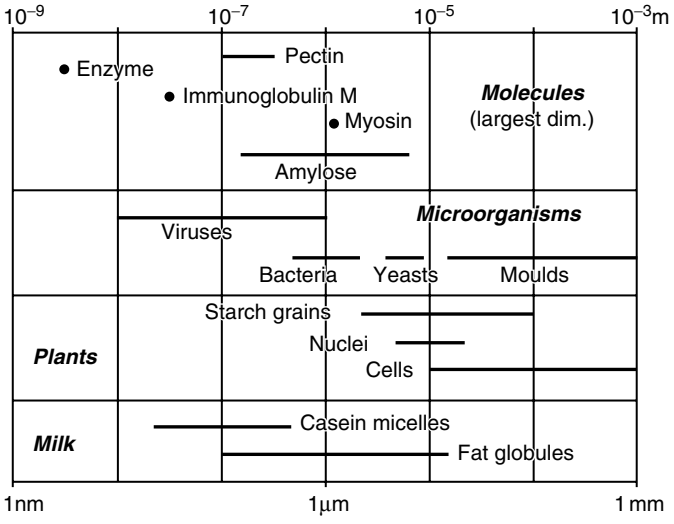

FIGURE 13.1 Approximate size of some structural elements in foods.

2. *Surface area*. For a collection of spheres each with a diameter d (in m), the specific surface area is given by

$$A = 6\frac{\phi}{d} \tag{13.1}$$

in $m^2\ m^{-3}$. The area can thus be large. For an emulsion of $\phi = 0.1$ and $d = 0.3\ \mu m$, $A = 2\ m^2$ per mL of emulsion; if 5 mg of protein is adsorbed per m^2 of oil surface, the quantity of adsorbed protein would amount to 1% of the emulsion.
3. *Pore size*. Between particles, regions of continuous phase exist and their size is proportional to particle size and smaller for a larger ϕ. If the dispersed phase forms a space-filling network, pores in this network follow the same rules. The permeability, that is, the ease with which solvent can flow through the pores, is proportional to pore size squared. This is why a polymer gel is far less permeable than a gel made up of fairly large particles (Section 13.5.2).
4. *Time scales involved*. (*Note*: Time scale is defined as the characteristic time needed for an event to occur; for instance, for two molecules to react, for a particle to rotate, for a bread to be baked.) The larger the particles, the longer are the time scales involved. For example, the root-mean-square value of the diffusion distance (z) of a particle of diameter d, as a function of time t is

$$\langle z^2 \rangle^{0.5} \propto \left(\frac{t}{d}\right)^{0.5} \tag{13.2}$$

In water, a particle of 10-nm diameter will diffuse over a distance equal to its diameter in about 1 μs; a particle of 1 μm in 1 s; and one of 0.1 mm in 12 days. Considering diffusion of a material into a structural element, the relation between diffusion coefficient D, distance l, and time $t_{0.5}$ needed to halve a difference in concentration is

$$l^2 \approx Dt_{0.5} \tag{13.3}$$

D of small molecules in water $\approx 10^{-9}\ m^2\ s^{-1}$ and in most cases (larger molecules, more viscous solution) it is smaller.
5. *Effect of external forces*. Most external forces acting on particles are proportional to diameter squared, whereas most attractive colloidal forces between particles are proportional to diameter. This implies that small particles are virtually impervious to external influences, like shearing forces or gravity. Large particles often can be deformed or even be disrupted by external forces. Large particles also sediment much faster.
6. *Ease of separation*. Some of the points raised above imply that it is much more difficult to separate small particles from a liquid than large ones.

Particles rarely are all of the same size. The subject of *size distributions* is a complicated one [2,66] and it will not be discussed here. Suffice it to say that a size range may generally be used to characterize the size distribution and that the volume/surface average diameter d_{vs} or d_{32} can often be seen as typical for the distribution. However, different properties may need different types of averages. The wider the size distribution—width being defined as standard deviation divided by average—the greater the differences between average types (an order of magnitude is not exceptional). It is often very difficult to accurately determine a size distribution [2]. Difficulties in determination and interpretation increase with particles that are more anisometric or otherwise different in properties.

13.1.3 Effects on Reaction Rates

As mentioned above, components in a dispersed food may be compartmentalized, and this can greatly affect reaction rates. In a system containing an aqueous (α) and an oil phase (β), a component often

is soluble in both. Nernst's distribution or partitioning law then states that the ratio of concentrations (c) in both phases is constant:

$$\frac{c_\alpha}{c_\beta} = \text{constant} \tag{13.4}$$

The constant will depend on temperature and possibly other conditions. For instance, pH has a strong effect on the partitioning of carboxylic acids, since these acids are oil soluble only when they are in a neutralized state. At high pH, where the acids are fully ionized, almost all of the acid will be in the aqueous phase, whereas at low pH the concentration in the oil phase may be considerable. Note that the quantity of a reactant in a phase also depends on the phase volume fraction.

When a reaction occurs in one of the phases present, the reaction rate does not depend on the overall concentration of a reactant, but on its concentration in the phase mentioned [93]. This concentration may be higher or lower than the overall concentration, depending on the magnitude of the partitioning constant (Equation 13.4). Since many reactions in foods actually are cascades of several different reactions, the overall reaction pattern, and thereby the mixture of components formed, may also depend on partitioning. Chemical reactions will often involve transport between compartments and will then depend on distances and molecular mobility. Applying Equation 13.3, it follows that diffusion times for transport into or out of fairly small structural elements, say emulsion droplets, would mostly be very short. However, if the solvent is immobilized in a network of structural elements, this may greatly slow down reactions, especially if reactants, say O_2, have to diffuse in from outside. Moreover, some reactions especially occur at the boundary between phases. An example is lipid autoxidation, where the oxidizable material (unsaturated oil) is in oil droplets, and a catalyst, say Cu ions, is in the aqueous phase. Another example is that of an enzyme present in one structural element and the component on which it acts in another one. In such cases, the specific surface area may be rate determinant. Adsorption of reactive substances onto interfaces between structural elements may diminish their effective concentration and thereby reactivity. Thus, rates of chemical reactions and the mixture of reaction products may be quite different in a dispersed system than in a homogeneous one. Examples in vegetable and animal tissues are well known, but other cases have not been studied in great detail, except for the activity of some additives [93] and, of course, enzymatic lipolysis of the oil in emulsion droplets.

13.2 SURFACE PHENOMENA

As mentioned above, most foods have a large phase boundary or interfacial area. Often, substances adsorb onto interfaces and this has a considerable effect on static and dynamic properties of the system. In this section basic aspects are discussed; applications are discussed later (e.g., see [1,3] for general literature).

Various types of interfaces can exist between two phases, the main ones being gas–solid, gas–liquid, liquid–solid, and liquid–liquid. If one of the phases is a gas (mostly air), one usually speaks of a surface, in the other cases of an interface, but these words are often considered to be interchangeable. More important is the distinction between a solid interface, where one of the phases is a solid and a fluid interface between two fluids (gas–liquid or liquid–liquid). A solid interface is rigid, a fluid interface can be deformed.

13.2.1 Interfacial Tension and Adsorption

An interface between two phases contains an excess of free energy, which is proportional to the interfacial area. Consequently, the interface will try to become as small as possible, to minimize the interfacial free energy. This then means that one has to apply an external force to enlarge the interfacial area. The reaction force in the interface is attractive and acts in the plane of the interface. If the interface is fluid, the force can be measured (see Figure 13.2a) and the force per unit length is

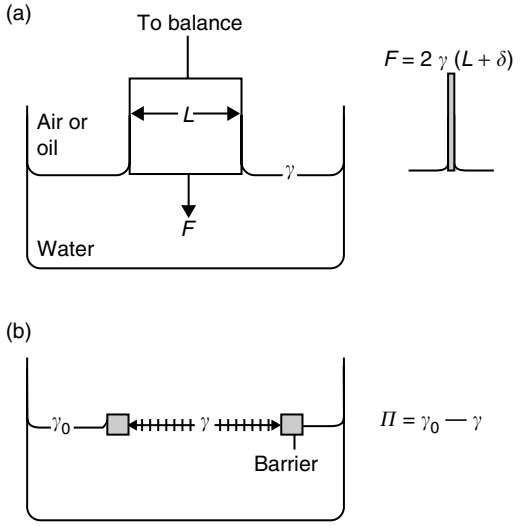

FIGURE 13.2 (a) Measurement of surface or interfacial tension by means of a Wilhelmy plate (width L, thickness δ). The plate is attached to a sensitive balance. F = net force. (b) Illustration of the surface pressure (Π) caused by adsorbed surfactant molecules (depicted by vertical dashes). Between the barriers the surface tension is lowered and a net two-dimensional pressure of magnitude Π acts on the barriers.

TABLE 13.1
Some Interfacial Tensions

Material	Against Air	Against Water
Water	72	0
Saturated NaCl solution	82	0
0.02 M SDS in water	41	0
Ethanol	22	0
Paraffin oil	30	50[a]
Triacylglycerol oil	35	30
Mercury	486	415

Note: Approximate values (mN m^{-1}) at room temperature.
[a] Some buffers give a lower interfacial tension than water.

called the surface or *interfacial tension*: symbol γ, units N m^{-1}. (γ_{OW} means the tension between oil and water, γ_{AS} between air and a solid, etc.) Also a solid has a surface tension, but it cannot be measured.

The magnitude of γ depends on the composition of the two phases. Some examples are given in Table 13.1. The interfacial tension also depends on temperature and it nearly always decreases with increasing temperature.

Some molecules in a solution that is in contact with a phase surface can accumulate at this surface, forming a monolayer. This is called *adsorption*. (*Note:* A*d*sorption is to be distinguished from a*b*sorption, where a substance is taken up *in* a material.) A substance that does adsorb is called a *surfactant*. It adsorbs because that gives a lower surface free energy, hence a lower surface tension. Examples are in Figure 13.3a. It is seen that the decrease in γ depends on the surfactant

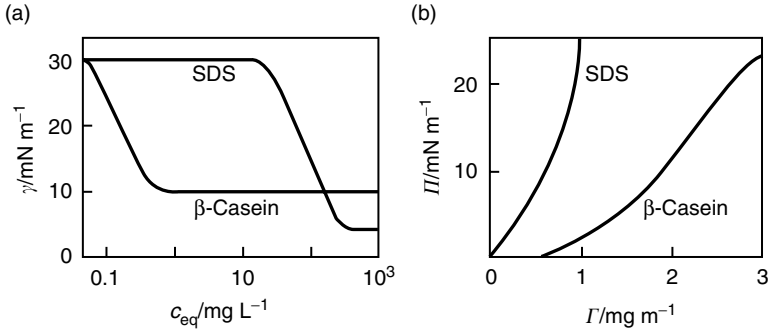

FIGURE 13.3 Absorption of β-casein and SDS at an oil–water interface. (a) Interfacial tension (γ) as a function of equilibrium surfactant concentration (c_{eq}). (b) Relation between surface pressure (Π) and surface load (Γ) (approximate results) (From Walstra, P., et al. (2006), *Dairy Science and Technology*, CRC/Taylor & Francis.)

concentration left in solution after equilibrium has been reached. The lower the value of c_{eq} at which a given decrease in γ is obtained, the higher the *surface activity* of the surfactant.

An important variable is the *surface load*, Γ, that is, the amount (in moles or in mass units) of adsorbed material per unit surface area. For $\Gamma = 0$, $\gamma = \gamma_0$, the value for a clean interface. At a relatively high surfactant concentration (c_{eq}), the value of Γ reaches a plateau, where the surfactant has made a packed monolayer. The plateau of Γ corresponds to the surfactant concentration at which γ reaches a plateau value. The magnitude of $\Gamma_{plateau}$ varies among surfactants, for the most part between 1 and 4 mg m^{-2}. The relation between Γ and the equilibrium surfactant concentration is called an *adsorption isotherm*. Substances in a gas phase, such as water in air, can also adsorb onto a (solid) surface, and the same relations apply.

Each surfactant has at equilibrium (and at a given temperature) a fixed relation between the magnitude of Γ and the decrease of γ. The latter is called the *surface pressure* $\Pi = \gamma_0 - \gamma$ (cf. Figure 13.2b). The maximum value of Π varies among surfactants; for many surfactants (though not for all) the value is roughly the same for air–water and oil–water interfaces. The relation between Π and Γ is called *surface equation of state*. Examples are given in Figure 13.3b.

The *rate of adsorption* of a surfactant depends primarily on its concentration. The surfactant will often be transported to a surface by diffusion. If its concentration is c and the surface load to be obtained Γ, a layer adjacent to the surface of thickness Γ/c will suffice to provide the surfactant. Application of Equation 13.3 and putting $l = \Gamma/c$ leads to

$$t_{0.5} = \frac{\Gamma^2}{Dc^2} \tag{13.5}$$

In aqueous solutions D is generally on the order of 10^{-10} m^2 s^{-1}. Assuming a surfactant concentration of 3 kg m^{-3}, and $\Gamma = 3$ mg m^{-2}, then results in $t_{0.5} \approx 10$ ms. Adsorption will be complete in about 10 times $t_{0.5}$, that is, well within a second. If the surfactant concentration is lower, adsorption will take a (much) longer time, but then stirring will markedly enhance adsorption rate. In other words, adsorption will nearly always be fast in practice.

13.2.2 Surfactants

Surfactants come in two main types, polymers and small amphiphilic molecules. (*Note on terminology*: Some authors use the word surfactant for small-molecule amphiphiles only. Also, surfactants are often called emulsifiers.)

13.2.2.1 Amphiphiles

The hydrophobic (lipophilic) part of a small-molecule amphiphile typically is an aliphatic chain. There is a wide diversity of hydrophilic parts. In the classical surfactant, common soap, it is an ionized carboxyl group. Most amphiphilic substances are not highly soluble either in water or oil, and they suffer the smallest repulsive interaction from these solvents when they are partly in a hydrophilic environment (water) and partly in a hydrophobic one (oil) (i.e., at an o/w interface) (see Figure 13.4); they also adsorb onto air–water and some solid–water interfaces. In solution, they tend to associate and form micelles to minimize repulsive interaction with solvent.

Some small-molecule surfactants of importance to the food scientist are listed in Table 13.2 [35,60]. They are categorized as nonionic, anionic, and cationic, according to the nature of the hydrophilic part. Also, distinction is made between natural surfactants (e.g., soaps, monoacylglycerols, phospholipids) and synthetic ones. The Tweens are somewhat different from other ones in that the hydrophilic part contains three or four poly(oxyethylene) chains of about five monomers in length. Phospholipids come in a wide range of composition and properties; several are zwitterionic.

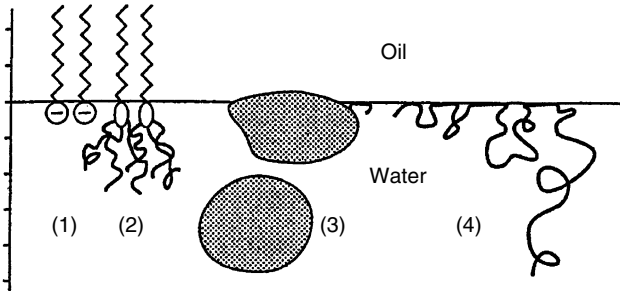

FIGURE 13.4 Mode of absorption of some surfactants at an oil–water interface; at left is a scale of nanometers. (1) A soap; (2) a Tween; (3) a small globular protein (for comparison a molecule in solution is shown); (4) β-casein. Highly schematic. (Walstra, P., et al. (2006), *Dairy Science and Technology*, CRC/Taylor & Francis.)

TABLE 13.2
Some Small-Molecule Surfactants and Their HLB Values

Type	Example of Surfactant	HLB Value
Nonionics		
Aliphatic alcohol	Hexadecanol	1
Monoacylglycerol	Glycerol monostearate	3.8
Esters of monoacylglycerols	Lactoyl monopalmitate	8
Spans	Sorbitan monostearate	4.7
	Sorbitan monooleate	7
	Sorbitan monolaurate	8.6
Tween 80	Poly(oxyethylene) sorbitan monooleate	16
Anionics		
Soap	Na oleate	18
Lactic acid esters	Na stearoyl-2-lactoyl lactate	21
Phospholipids	Lecithin (zwitterionic)	Fairly large
Teepol[a]	SDS	40
Cationics[a]		Large

[a] Not used in foods but as detergents.

An important characteristic of a small-molecule surfactant is its *HLB value*, where HLB stands for hydrophile–lipophile balance. It is defined so that a value of 7 means that the substance has about equal solubility in water and oil. Smaller values imply greater solubility in oil, and so forth. Surfactants range in HLB value from about 1 to 40. The relation between HLB value and solubility is in itself useful, but it also relates to the suitability of the surfactant as an emulsifier: surfactants with HLB >7 are generally suitable for making foams and o/w emulsions, and those with HLB <7 for w/o emulsions (see also Section 13.6.2 about Bancroft's rule). Surfactants suitable as cleaning agents (detergents) in aqueous solution have a large HLB number. Several other relations with HLB values have been claimed, but most of these are questionable.

Originally, the HLB value of a surfactant was determined from its solubility in water divided by that in oil. Currently, HLB numbers have been derived for a range of chemical groups. Several authors have given tabulated values (e.g., [27]). The polar group(s) of a surfactant have a positive value and the hydrophobic groups have a negative value. The sum of these values plus 7 give the HLB number. In general, a longer aliphatic chain yields a lower HLB, and a more polar or a larger polar group a higher HLB. Actually, the HLB number of a surfactant will depend on temperature and on oil type.

As mentioned above, many small-molecule amphiphiles tend to form micelles, which occurs above a *critical micellization concentration* (CMC). Beyond that concentration additional surfactant molecules will go into micelles and their thermodynamic activity (or effective concentration, roughly speaking) barely increases. Consequently, the surface load Γ does not further increase and γ does not further decrease. In Figure 13.3a, the CMC for sodium dodecyl sulfate (SDS) is thus reached at a total concentration of about 300 mg L^{-1}. In a homologous series of surfactants, a longer chain length results in a smaller value of the CMC. For ionic surfactants, the CMC markedly decreases with increasing ionic strength. The CMC can also depend on pH.

At the air–water interface much the same pattern is observed, but since γ_0 is higher and Π is roughly the same, γ is much higher. The smallest value of γ obtained at an air–water interface is about 35 mN m^{-1}, whereas at a triacylglycerol oil–water interface it varies from <1 to about 5 mN m^{-1} for most small-molecule surfactants.

It should be realized that commercially available surfactants generally are *mixtures* of several components, varying in chain length and possibly in other properties. These components may differ, for example, in the plateau value of γ. Especially, some trace components may be present that give a lower γ than the main components, and at equilibrium the surfactants yielding the smallest γ will dominate in the interface. Because of their smaller concentration, however, their diffusion to the interface will be slow (see Equation 13.5). This implies that it will take a long time before an equilibrium composition, and thus a steady γ-value, is reached. Another complication is that in actual dispersions the surface-to-volume ratio is very large, whereas this ratio is quite small in situations where γ is commonly measured (i.e., at a macroscopic interface between the phases). This means that the result of such measurements of γ may not be representative for the actual values in a foam or emulsion.

13.2.2.2 Polymers

Several synthetic polymers can be used as surfactants and a mass of experimental evidence as well as theory is available [26]. Copolymers, where part of the segments are fairly hydrophobic and others hydrophilic, are suitable. They tend to adsorb with "trains," "loops," and "tails" (cf. Figure 13.4, curve 4). There are few natural polymers that adsorb in this way. Surface activity of polysaccharides still is controversial. Most surface active polysaccharides contain a protein moiety that is responsible for this attribute [20]. On the other hand, chemical modification can provide polysaccharides with hydrophobic groups; a well-known example is given by some cellulose ethers, which can be used as emulsifiers [13].

Proteins often are the surfactants of choice in food technology, especially for foams and o/w emulsions [18,53,87]. (Because of their insolubility in oils, they are not suitable for w/o emulsions.) The mode of adsorption of proteins varies (cf. Figure 13.4). There always is a change in conformation, often considerably so. For instance, most enzymes (with the exclusion of true lipases) completely lose their activity after adsorption at an oil–water interface due to conformational change; some enzymes retain part of their activity after adsorption at an air–water interface [16]. Most globular proteins appear to retain an approximately globular conformation at interfaces, though not the native one. Proteins with little secondary structure, such as gelatin and caseins, tend to adsorb more like a linear polymer. This implies that they protrude much farther into the aqueous phase than most globular proteins. The latter can be denatured prior to absorption (e.g., by heat treatment), which alters their conformation after adsorption; generally, Γ and the protrusion distance are increased. At high bulk protein concentration, multilayer adsorption may occur, but the second and more remote layers are only weakly adsorbed.

In Figure 13.3, adsorption of a protein and an anionic surfactant are compared and there are three main differences that generally hold for proteins and synthetic high polymers, as compared to small-molecule amphiphiles:

1. The protein is clearly more surface active than the anionic. Consequently, desorption of adsorbed proteins cannot or barely be achieved by dilution or "washing." The difficulty of desorption may be enhanced by cross-linking reactions between adsorbed protein molecules; this has especially been shown for proteins containing a free thiol group, where cysteine–cystine interchange can occur in the interface [24].
2. As shown in Figure 13.3b, the surface equation of state differs greatly between the protein and the SDS. For a protein, the value of Γ at which a significant surface pressure observed is much higher than for an amphiphile. This is because at low values of Γ, the magnitude of Π is proportional to the surface load expressed in moles of surfactant per unit of interfacial area, taking into account that the molar mass of a typical protein is about 100 times that of a typical amphiphile. This has some important consequences for emulsion and foam making (Sections 13.6.2 and 13.7.1).
3. The anionic surfactant yields a lower interfacial tension than the protein at the plateau adsorption. The magnitude of the interfacial tension affects several phenomena, as discussed later on. Here, we will mention one aspect, that is, the displacement of a protein from the interface by an amphiphile that is present in a high concentration [15]. It is illustrated in Figure 13.5. Many foods naturally contain some surfactants (fatty acids, monoacylglycerols, and phospholipids) and these can modify the properties of adsorption layers.

To some extent, proteins can also displace each other in a surface layer, depending on concentration, surface activity, molar mass, molecular flexibility, and so forth. Although protein adsorption appears irreversible in the sense that it is mostly not possible to substantially lower Γ by diluting the system, the occurrence of mutual displacement nevertheless implies that individual protein molecules in the interfacial layer may interchange with those in solution, albeit slowly.

13.2.3 Contact Angles

When two fluids are in contact with a solid and with each other, there is a contact line between the three phases [1]. An example is given in Figure 13.6a for the system air–water–solid. There must be a balance between the surface forces acting in the plane of the solid surface and this leads to the Young equation

$$\gamma_{AS} = \gamma_{WS} + \gamma_{AW} \cos\theta \tag{13.6}$$

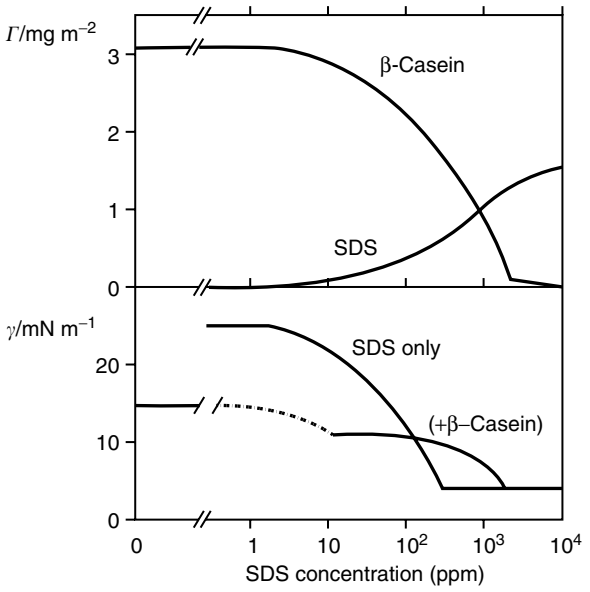

FIGURE 13.5 Surface load (Γ) in an o/w emulsion, and interfacial tension (γ) at the o/w interface, for β-casein in the presence of increasing concentration of SDS; γ is also given for SDS only. (From results in Walstra, P. and A.L. de Roos (1993), *Food Rev. Intern.* **9**: 503—525.)

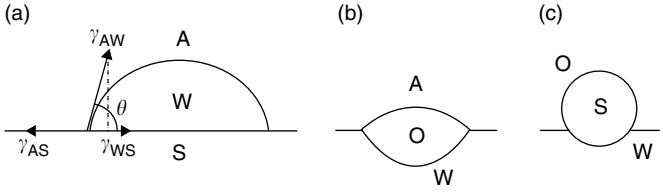

FIGURE 13.6 Contact angles (θ): examples of three-phase systems. A = air; O = oil; S = solid; W = water. The arrows in (a) indicate the values of the three interfacial tensions. (Walstra, P., et al. (2006), *Dairy Science and Technology*, CRC/Taylor & Francis.)

The contact angle θ is conventionally taken in the densest fluid phase. Its value depends on three interfacial tensions. γ_{AS} and γ_{WS} cannot be measured, but their difference can be derived from the contact angle. If $(\gamma_{AS} - \gamma_{WS})/\gamma_{AW} > 1$, Equation 13.6 has no solution, $\theta = 0$, and the solid will be completely wetted by the liquid; an example is water on clean glass. If the quotient mentioned is <-1, there is no wetting at all; an example is water on Teflon or other strongly hydrophobic materials.

In Figure 13.6b the more complicated situation of contact between three fluids is shown. Now there has to be a balance of surface forces in the horizontal as well as in the vertical plane, giving two contact angles. A spreading pressure can be defined as

$$\Pi_S = \gamma_{AW} - (\gamma_{AO} + \gamma_{OW}) \tag{13.7}$$

In Figure 13.6b, $\Pi_S < 0$. If it is >0, the sum of the surface free energies of the A/O and the O/W interfaces is smaller than that of the A/W interface alone, and the oil will spread over the water surface. Use of the values in Table 13.1 leads to the conclusion that for paraffin oil $\Pi_S = -8\ \text{mN m}^{-1}$, implying that the droplet will not spread (but it does adhere to the a/w interface). For triacylglycerol

oil, it follows that $\Pi_S = 7$ mN m^{-1}, and spreading will occur. These aspects are of importance for the interactions between emulsion droplets and foam bubbles. The spreading pressures can, of course, be altered by surfactants. However, most proteins lower γ_{AW} and γ_{OW} by roughly the same amount; therefore, the spreading pressure is not greatly altered.

Figure 13.6c depicts a small solid particle located in an oil–water interface. The Young equation also applies in this case. The contact angle (about 140° in the water phase) would be fairly typical for a triacylglycerol crystal in a triacylglycerol oil–water interface. The contact angle can in such a case be lowered by adding a suitable surfactant (e.g., SDS) to the water phase. Addition of a large quantity of surfactant can even lead to $\theta = 0$, and thus to complete wetting of the crystal by the aqueous phase. This is accomplished in some processes to separate fat crystals from oil. Adherence of crystals to the o/w interface and the associated contact angle may be of importance for emulsion stability (e.g., Section 13.6.5).

It should be noted that the action of gravity can alter the shape of the fluid interfaces depicted in Figure 13.6, but the contact angles remain the same. If the droplets are smaller than about 1 mm, the effect of gravity tends to be quite small.

13.2.4 Curved Interfaces [1]

The pressure at the concave side of a curved phase boundary (interface) is always greater than that at the convex side. The difference is called the *Laplace pressure* p_L, given by

$$p_L = \frac{2\gamma}{R} \tag{13.8}$$

where R is the radius of curvature; for a spherical particle, R equals the particle radius r.

An important consequence is that drops and bubbles tend to be spherical and that it is difficult to deform them, the more so when they are smaller. If a drop is not spherical, the radius of curvature differs with location, which implies a pressure difference within the drop. This causes material in the drop to move from regions with a high pressure to those with a lower one, until a spherical shape is obtained. Only if an external stress is applied, can the drop (or bubble) be deformed from the spherical shape. Some examples may be enlightening. For an emulsion droplet of radius 0.5 μm and interfacial tension 0.01 N m^{-1}, the Laplace pressure would be 4×10^4 Pa (0.4 bar) and a considerable external pressure would be needed to cause substantial deformation. For an air bubble of 1 mm radius and $\gamma = 0.05$ N m^{-1}, p_L would be 100 Pa, allowing deformation to occur more easily. These aspects will be discussed further in Sections 13.6.2, 13.6.4, and 13.7.1.

Another consequence of the Laplace pressure is *capillary rise*, illustrated in Figure 13.7a. In a vertical capillary containing a liquid that gives zero contact angle (e.g., water in a glass tube), a concave meniscus is formed. For a capillary of radius r, this implies a pressure difference of

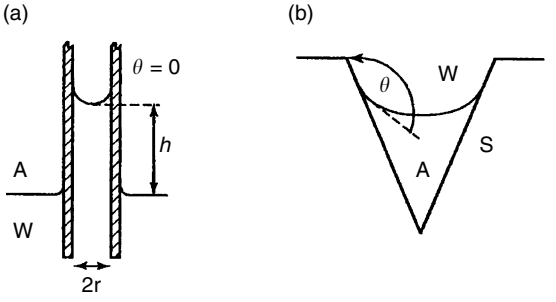

FIGURE 13.7 Some capillary phenomena. (a) Rise of a liquid in a capillary if the contact angle $\theta = 0$. (b) Air pocket in a crevice in a submerged solid in water (see text).

magnitude $2\gamma/r$ between the water just below the meniscus and that outside the tube at the same height. The liquid in the tube will then rise, until the pressure due to gravity ($g\ \rho\ h$) balances the capillary pressure. For example, pure water in a cylindrical capillary of 0.1 mm internal radius would rise 15 cm. If the contact angle is larger, the rise will be less; if it is >90°, capillary depression occurs.

These aspects are relevant to the dispersion of powders in water. If a heap of powder is placed on water, capillary rise of the water through the pores (voids) between the powder particles must occur for wetting of the particles to occur, and this is a prerequisite for dispersion, hence dissolution, of the powder. It requires a contact angle (between powder material, water, and air) <90°. The effective contact angle in a powder is substantially larger than that at a smooth surface of the powder material, so the latter angle must be distinctly smaller than 90° for wetting of the powder to occur (see [73]).

A third consequence of Laplace pressure is the *enhanced solubility* of the gas in a bubble in the liquid around it. According to Laplace (Equation 13.8), the pressure of a gas in a (small) bubble is enhanced and, according to Henry's law, the solubility of a gas is proportional to its pressure. The effect of curvature of a particle on the solubility of the material in the particle is not restricted to gas bubbles and is in general given by the *Kelvin equation*

$$RT \ln \frac{s(r)}{s_\infty} = \frac{2\gamma M}{\rho r} \tag{13.9}$$

for a spherical particle of radius r; s is solubility, s_∞ solubility at a plane interface (i.e., "normal" solubility), and M and ρ are molar mass and mass density, respectively, of the material in the particle. Examples of calculations according to Equation 13.9 are in Table 13.3. It is seen that for most systems, particle radius has to be very small (e.g., <0.1 μm) for a significant effect. However, gas in bubbles of 1 mm has a perceptibly enhanced solubility.

The increased solubility gives rise to *Ostwald ripening*, that is, the growth of large particles in a dispersion at the expense of small ones, and thus the eventual disappearance of the smallest particles. However, this only occurs if the material of the particles is at least somewhat soluble in the continuous phase. It may thus occur in foams and in w/o emulsions, but not in triacylglycerol o/w emulsions. The rate of Ostwald ripening is governed by several factors (e.g., see Section 13.7.2).

Ostwald ripening will always occur with crystals in a saturated solution, albeit slowly if the crystals are large. Another effect is that it causes "rounding" of small crystals. At the edge of a crystal, the radius of curvature may be very small, say some nanometers, and this will lead to a greatly enhanced solubility (Table 13.3, fat crystal in oil). The material near the edge will thus dissolve and be deposited somewhere else. Small ice crystals (say, 20 μm) in partly frozen foods are generally of a fairly isometric shape.

If the surface of a particle is (partly) concave rather than convex, as shown in Figure 13.7b, the solubility is, of course, decreased. If the situation depicted represents a local equilibrium, the gas

TABLE 13.3
Examples of the Increase in Solubility of the Material in a Particle Due to Curvature[a]

Variable	Water in Oil	Air in Water	Fat Crystal in Oil	Sucrose Crystal in Saturated Solution
R (m)	10^{-6}	10^{-4}	10^{-8}	10^{-8}
γ (N m^{-1})	0.005	0.05	0.005	0.005
ρ (kg m^{-3})	990	1.2	1075	1580
M (kg mol^{-1})	0.018	0.029	0.70	0.342
s_R/s_∞	1.000073	1.010	1.30	1.091

[a] Calculated according to Equation 13.9 for some arbitrary radii of curvature and some reasonable values of the interfacial tension (temperature 300 K).

concentration in the liquid is below saturation. If the gas concentration would be higher, the gas pocket depicted will grow.

13.2.5 Interfacial Rheology [4,5,39,87]

If an interface contains surfactant, it has rheological properties. Two kinds of surface rheology can be distinguished, in shear and in dilation (Figure 13.8). When the interface is sheared (leaving both area and amount of surfactant in the interface constant), one can measure the force in the plane of the interface needed to do this. Often this is done as a function of the shear rate, and a *surface shear viscosity* η_{SS} (units N s m^{-1}) is obtained. For most surfactants η_{SS} is negligibly small, but not for several polymeric surfactants. For example, for a Na-caseinate monolayer, a viscosity of 0.002 N s m^{-1} has been observed, for layers of globular proteins values between 0.01 and 1 N s m^{-1}. For most systems, shear rate thinning occurs and the observed viscosity is an apparent viscosity. Values reported for globular proteins greatly vary, partly because of experimental uncertainty: the monolayer can yield or rupture, and the measured "viscosity" then will greatly depend on the rupture pattern [44]. For some proteins, the viscosity strongly increases with the age of the monolayer, due to the formation of intermolecular bonds [16].

If the interfacial area is enlarged, leaving its shape unaltered, an increase in interfacial tension will occur, because Γ is decreased. This is usually expressed in the *surface dilational modulus*, defined as

$$E_{SD} \equiv \frac{d\gamma}{d \ln A} \tag{13.10}$$

where A is the interfacial area. E_{SD} is finite for all surfactants, although it will be very small if surfactant activity is high and the rate of surface enlargement is small. In such a case, surfactant from the bulk rapidly diffuses to the enlarged surface, thereby increasing Γ and lowering γ. In other words, the equilibrium between bulk (c_{eq}) and interfacial concentration (Γ) will be rapidly restored. E_{SD}, therefore, strongly decreases with decreasing rate of deformation. For proteins E_{SD} may be large and less dependent on time scale, because proteins adsorb more or less irreversibly. However, the interfacial concentration of protein has a large effect: Figure 13.3b shows that for a protein the value of Γ has to be high before a significant value of Π, hence of E_{SD}, is attained. Changes in protein conformation upon adsorption and dilation can also affect the modulus.

E_{SD} is a property that appears in several equations relating to interfacial phenomena. A problem is, however, that the measurement of E_{SD} is difficult or even impossible, except at relatively long time scales and/or small deformations. By and large, for globular proteins at the a/w interface, values of about 30–100 mN m^{-1} have been observed and for β-casein about 10–20 mN m^{-1} [4,29,52,71]. Values at the o/w interface may be significantly different from those at the a/w interface.

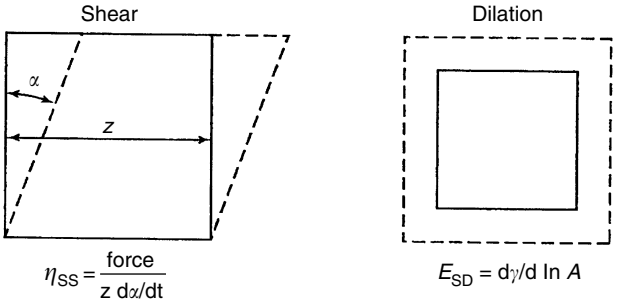

FIGURE 13.8 Illustration of the geometrical changes applied in a surface element when performing surface rheology in simple shear and in dilation.

Surface rheological parameters of protein layers naturally depend on pH, ionic strength, solvent quality, temperature, and so forth. Often moduli and viscosities are at maximum near the isoelectric pH. It may further be noted that one can also measure a surface dilational viscosity and a surface shear modulus.

13.2.6 Surface Tension Gradients

If a fluid interface contains a surfactant, surface tension gradients can be created. This is illustrated for the case of an a/w interface in Figure 13.9. In (a) a velocity gradient ($\nabla v = \mathrm{d}v_y/\mathrm{d}x$) sweeps surfactant molecules downstream, thereby producing a surface tension gradient: γ will now be smaller downstream. This implies that the surface exerts a *tangential stress* $\Delta\gamma/\Delta x$ onto the liquid. When the gradient is large enough, the stress can be equal and opposite to the shear stress $\eta \cdot \nabla v$ ($\eta =$ viscosity of the liquid), which means that the surface will not move. If there were no surfactant, the surface would move with the flowing liquid; in the case of an o/w interface, the flow velocity would be continuous across the interface.

This has important consequences, especially for foams, as is seen by comparing Frames (c) and (d). In the absence of a surfactant, the liquid between two foam bubbles rapidly streams downwards, like a falling drop. In the presence of surfactant, flow is very much slower as the "walls" of the film can now withstand the stress caused by the down flowing liquid. In other words, development of surface tension gradients is essential for the formation of a foam. It also means that a small air bubble or an emulsion droplet moving through the surrounding liquid in nearly all cases has an immobile surface, that is, it behaves like a rigid particle. In these cases, the γ-gradient can be quite large because Δx is small.

Figure 13.9b illustrates that liquid adjacent to an interface will move with the interface when the latter exhibits (for some reason, say local adsorption of surfactant) an interfacial tension gradient. This is called the *Marangoni effect*. It is seen in a glass of wine, where wine drops above the liquid level tend to move upward; here, evaporation of ethanol causes a local increase in γ, thereby producing a γ-gradient.

An important consequence of the Marangoni effect is that it provides stability to a thin film, as illustrated in Figure 13.9e. If the film acquires somehow a thin spot, the surface area of the film is locally increased, hence Γ is lowered, γ is increased, and a γ-gradient is established. This causes

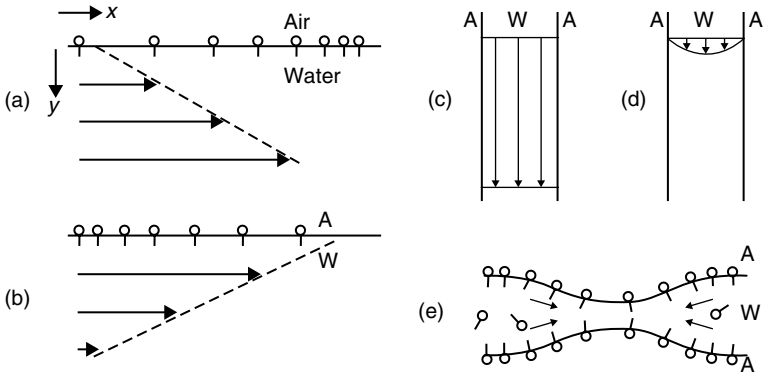

FIGURE 13.9 Surface-tension gradients at the a/w interface. (a) Streaming of liquid along a surface causes a surface-tension gradient. (b) Marangoni effect: a surface-tension gradient cause streaming of the adjacent liquid. (c) Drainage of liquid from a vertical film in the absence or (d) presence of surfactant. (e) Gibbs mechanism of film stability. (After Walstra, P. (1989), in *Foams: Physics, Chemistry and Structure* (A.J. Wilson, ed.), Springer, London, pp. 1–15.)

adjacent liquid to flow to the thin spot, thereby restoring film thickness. This "Gibbs mechanism" explains the stability of thin liquid films, as in a foam.

Interfacial tension gradients are also paramount in preventing coalescence of newly formed drops during emulsification, as discussed in Section 13.6.2. In all these situations, the effects depend on film or Gibbs elasticity, which is defined as twice the surface dilational modulus (twice because a film has two surfaces). Thin films typically have a large elasticity, because of the scarcity of dissolved surfactant. In a thick film containing a fairly high concentration of surfactant, surfactant molecules can rapidly diffuse towards a spot with a low surface load and restore the original surface tension. This cannot, or only very slowly, occur in a thin film, implying a large elasticity, except at quite long time scales.

13.2.7 Functions of Surfactants

Surfactants in a food, whether small-molecule amphiphiles or proteins, can produce several effects and these are briefly summarized below:

1. Due to the lowering of γ, the Laplace pressure is lowered and the interface can be more easily deformed. This is important for emulsion and foam formation (Section 13.6.2) and for the occurrence of coalescence (Section 13.6.4).
2. Contact angles are affected, which is important for wetting and dispersion events. The contact angle determines whether a solid particle can adsorb on a fluid interface and to what extent it then sticks out in either fluid phase. These aspects have an important bearing on stability of some emulsions (Section 13.6.5) and foams (Section 13.7.2).
3. A decrease in interfacial free energy will proportionally slow Ostwald ripening. The rate of Ostwald ripening may also be affected by the surface dilational modulus (Section 13.7.2).
4. The presence of surfactants allows the creation of surface tension gradients and this may be their most important function. It is essential for formation and stability of emulsions and foams (Sections 13.6.2, 13.6.4, 13.7.1, and 13.7.2).
5. Adsorption of surfactants onto particles may greatly modify (colloidal) interparticle forces, mostly enhancing repulsion and thereby stability. This is discussed in Section 13.3.
6. Small-molecule amphiphiles can form micelles, which can harbor some hydrophobic molecules, say oil molecules, in their interior. This greatly enhances the apparent solubility of several hydrophobic substances and forms the basis of detergency.
7. Small-molecule surfactants may undergo specific interactions with macromolecules. Ionic amphiphiles often associate with proteins, thereby materially altering some protein properties (e.g., isoelectric pH, apparent solubility, surface activity). Another example is the interaction of some lipid-like surfactants with amylose.

13.3 COLLOIDAL INTERACTIONS

In Section 13.1.2 colloids were defined and classified. Generally, between particles forces act that originate from material properties of the particles and the interstitial fluid. These colloidal interaction forces act in a direction perpendicular to the particle surface, contrary to the surface forces discussed in Section 13.2, which act in the plane of the surface. Both attractive and repulsive forces can act.

The net interaction force acting between colloidal particles has important consequences:

1. It determines whether particles will aggregate (Section 13.4.3), which, in turn, may determine further physical instability. For instance, aggregation of particles may lead to increased sedimentation, hence to a rapid formation of a cream layer or sediment. (*Note on terminology*: The terms flocculation and coagulation are also used, often with a more

specific connotation; the former would, for instance refer to reversible aggregation, the latter to irreversible.)

2. In other situations, aggregating particles may form a space-filling network, hence a gel (Section 13.5), and the rheological properties and the stability of systems containing such a network strongly depends on colloidal interaction.
3. The interaction forces greatly affect susceptibility of emulsion droplets and gas bubbles to coalescence, and also partial coalescence of fat globules (Sections 13.6.4 and 13.6.5).

The net effect of colloidal interactions can also depend on external forces, for example, due to gravity or agitation or an electric potential gradient, and on size and shape of the particles. Furthermore, the adsorption of surfactants on the particles may greatly modify the strength of repulsive forces.

We will briefly discuss some aspects of colloidal interactions, generally for the simple cases of identical spheres. Literature on colloid science can be found in textbooks mentioned in Further Reading.

13.3.1 van der Waals Attraction

van der Waals forces between molecules are ubiquitous and they also act between larger entities such as colloidal particles. Since these forces are additive, it turns out that, within certain limits, the dependence of the interaction force on interparticle distance (as measured between the outer surfaces) is much weaker between particles than between molecules. For two identical spherical particles the van der Waals interaction free energy is given by

$$V_A \approx \frac{Ar}{12h}, \quad h <\sim 10\ \text{nm} \tag{13.11}$$

where r is particle radius, h is interparticle distance, and A is the Hamaker constant. The latter depends on the material of the particles and of the fluid in between, and it increases in magnitude as the differences in the properties of the two materials increase. For most particles in aqueous foods, A is between 1 and 1.5 times kT ($kT \approx 4 \times 10^{-21}$ J at room temperature), but for air bubbles in water A is much larger, about 10 times kT. Tabulated values are available [31,42,43,79].

If both particles are of the same material and the fluid in between is different, A always is positive and the particles attract each other. If the two particles are of different materials, A may be negative, and there would be van der Waals repulsion, but this is fairly uncommon.

13.3.2 Electric Double Layers

Most particles in an aqueous solution exhibit an electric charge, because of adsorbed ions or ionic surfactants. In most foods, charges predominantly are negative. Since the system must be electroneutral, the particles are accompanied by a cloud of oppositely charged ions, called counterions. An example of the distribution of counterions and coions is shown in Figure 13.10a. It is apparent that at a certain distance from the surface, the concentrations of positive and negative charges in the solution become equal. Beyond that distance, the charge on the particle is neutralized, due to an excess of counterions in the electric double layer. The latter is defined as the zone between the particle surface and the plane at which neutralization is achieved. The double layer should not be envisaged as being immobilized, because solvent molecules and ions diffuse in and out of the layer.

The electrical effects are usually expressed in the electric potential ψ (in volts). Its value, as a function of the distance h from the surface, is given by

$$\psi = \psi_0 \exp(-\kappa h) \tag{13.12}$$

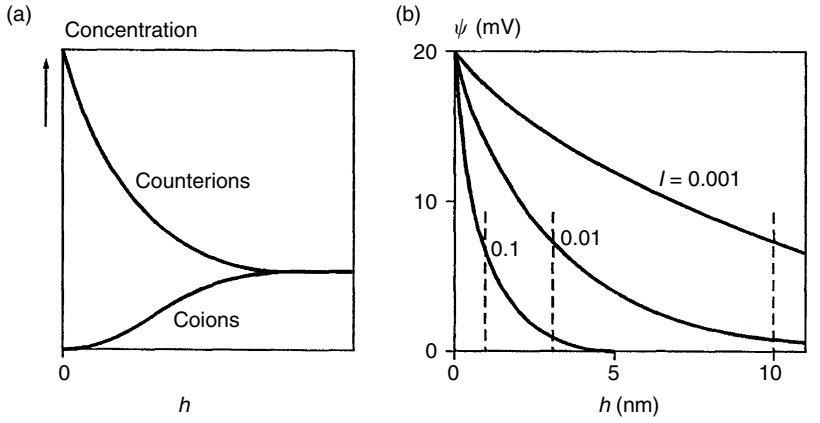

FIGURE 13.10 The electric double layer. (a) The distribution of counterions and coions as a function of the distance h from the charged surface. (b) The potential ψ as a function of distance for three values of the ionic strength I (mM); the broken lines indicate the Debye length $(1/\kappa)$.

where ψ_0 is the potential at the surface and the nominal thickness of the electric double layer or Debye length $1/\kappa$ is given by

$$\kappa \approx 3.2\, I^{0.5}\ (\mathrm{nm}^{-1}) \tag{13.13}$$

for dilute aqueous solutions at room temperature. The ionic strength I depends on the total ion concentration and is defined as

$$I \equiv \frac{1}{2}\Sigma m_i z_i^2 \tag{13.14}$$

where m is molar concentration and z is valence of each of the ionic species present. Note that for a salt like NaCl I equals the molarity of the solution, but this is not so if ions of higher valence are present. For $CaCl_2$, I is three times the molarity.

Calculations of the potential as a function of distance are in Figure 13.10b. Ionic strengths in aqueous foods vary from 1 mM (a typical tap water) to more than 1 M (pickled foods). The I-value of milk is about 0.075 M and of blood is about 0.14 M. Consequently, the thickness of the double layer is often only about 1 nm or less.

Electrical interactions depend on the surface potential and this, in turn, is generally dependent on pH. For most food systems, values of $|\psi_0|$ are below 30 mV. At a high concentration of counterions (especially if these are divalent), ion pairs can be formed between counterions and charged groups on the particle surface, thereby lowering $|\psi_0|$.

In a nonaqueous phase, the dielectric constant generally is much smaller than in water, and Equation 13.12 is no longer valid. Moreover, the ionic strength in this situation generally will be negligible. This means that even if there is a charged surface (as may be the case for aqueous droplets floating in oil), electrical interactions forces will generally be unimportant.

13.3.3 DLVO Theory

If electrically charged particles having the same sign come very close to each other, their electric double layers overlap. This is sensed and the particles repulse each other. The repulsive electric interaction free energy V_E can be calculated. For spheres of equal size it is in first approximation given by the proportionality

$$V_E \propto r\psi_0^2 \exp(-\kappa h) \tag{13.15}$$

The interaction energies V_A (due to van der Waals attraction) and V_E can be added and this has led to the first useful theory for colloid stability, the DLVO (Deryagin–Landau, Verwey–Overbeek)

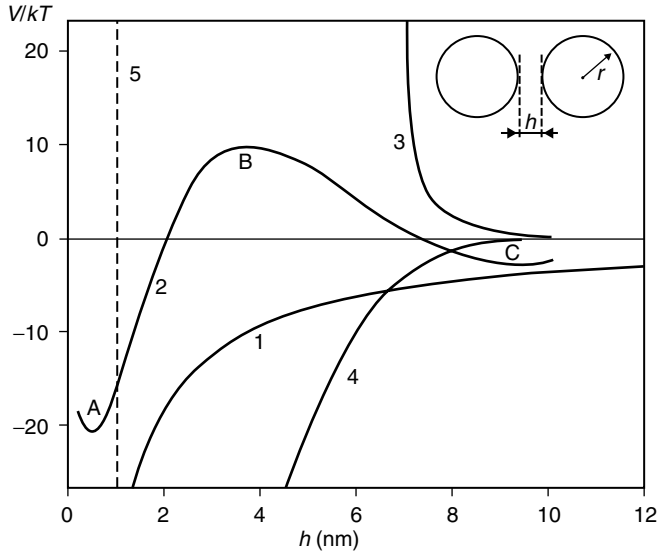

FIGURE 13.11 Calculated examples of the interaction free energy V between two particles as a function of their separation distance h; the inset shows the geometry considered. Curves (1) van der Waals attraction; (2) DLVO-interaction; (3) steric repulsion; and (4) depletion interaction (see text for further explanation). (Walstra, P., et al. (2006), *Dairy Science and Technology*, CRC/Taylor & Francis.)

theory. This theory enables calculation of the total free energy V needed to bring two particles from infinite distance to a distance h. We will not elaborate on this because accurate calculation is generally not possible for food systems. The total interaction energy is usually divided by kT, that is, the average kinetic energy involved in an encounter between two particles by Brownian (heat) motion.

Some trends will be discussed with reference to Figure 13.11. Curve 1 gives an example of van der Waals attraction. It always becomes stronger for a smaller interparticle distance. Curve 2 is an example of the sum of van der Waals attraction and electrostatic repulsion. In this case there is a so-called secondary minimum in the curve near C. Brownian motion will then readily cause a pair of particles to reach this point. The value of V is here about $-3kT$, sufficient to cause aggregation of the particles. However, the particles can deaggregate again, because the net attraction is only weak in this example. The particle pair may even occasionally overcome the free energy maximum at point B (about $10kT$), which implies that they then will reach the primary minimum A, which is so deep that the particles become permanently aggregated. Equation 13.11 would even predict that V_A would go to $-\infty$ at $h \to 0$, but at very small h hard-core repulsion between the atoms in the surface layer of the two particles will prevent this.

Generally, it is not possible to alter van der Waals attraction, but electrostatic repulsion can be readily modified. Lowering of the ionic strength causes the repulsion to act over a greater distance and will virtually remove the secondary minimum. An increase of the charge on the particles, hence of $|\psi_0|$, either by adding an ionic surfactant or by manipulating the pH, causes especially the maximum in the curve to increase, thereby preventing permanent aggregation. If both $|\psi_0|$ is low and I is high, attraction will prevail over all distances and the particles will rapidly aggregate.

Generally, even though the DLVO theory is very successful for many inorganic systems, it is inadequate for predicting stability of most biogenic systems. Milk fat globules, for example, are stable against aggregation at their isoelectric pH (3.8), where they have zero surface potential, so that the DLVO theory would predict zero repulsion [88]. Consequently, interaction forces other than those considered in this theory must be important.

13.3.4 Steric Repulsion

As depicted in Figure 13.4, some adsorbed molecules (polymers, Tweens, etc.) have flexible molecular chains ("hairs") that protrude into the continuous phase. These may cause steric repulsion. Two mechanisms can be distinguished. First, if the surface of another particle comes close, the hairs are restricted in the conformations they can assume, which implies loss of entropy, hence increase of free energy, and repulsion occurs. This volume-restriction effect can be very large, but it can be of importance only if the surfaces have a very low hair density (number of hairs per unit area). This is because the hairy layers start to overlap on approach of the particles and then a second mechanism will act before the first one comes into play. The overlap causes an increased concentration of protruding hairs and thereby an increased osmotic pressure; this then leads to water moving to the overlap region, which results in repulsion. However, this is true only if the continuous phase is a good solvent for the hairs; if it is not, attraction may result. For example, emulsion droplets covered by casein have protruding hairs, providing stability to the droplets. If ethanol is added to the emulsion, the solvent quality is strongly decreased and the droplets aggregate [18].

In some cases, steric repulsion free energy can be calculated with reasonable accuracy [26]. If these values are added to the van der Waals attraction, curves for total interaction vs. interparticle distance are obtained. The solvent quality usually is of overriding importance and if it is good, repulsion can be very strong (cf. curve 3 in Figure 13.11). In practical food systems calculation of steric repulsion usually is not possible because the situation is too complex. For instance, the nature of the adsorbing molecules can vary greatly [21,26,83]. An example is proteins that exhibit protruding hairs upon adsorption, such as the caseins; these hairs carry electric charges that can increase repulsion.

On the other hand, adsorbing polymers may cause bridging aggregation, when becoming simultaneously adsorbed onto two particles [26,83]. This may happen if too little polymer is present to fully cover the particle surface area or with certain methods of processing. Moreover, interparticle bonds may be formed between adsorbed proteins, for example, due to —S—S— bridge formation at high temperatures, or —Ca— bridges between negative charges on the hairs if sufficient Ca^{2+} is present. Altogether, subtle changes in composition of an aqueous dispersion may have a profound effect on colloidal stability.

13.3.5 Depletion Interaction

Besides polymer chains protruding from a surface, polymer molecules in solution can affect colloidal interaction. Consider a liquid dispersion, for example, an emulsion, that also contains some (nonadsorbing) dissolved polymer, say, xanthan. The centre of a polymer molecule cannot come closer to the surface than a value δ, which is about equal to its radius R_g, as illustrated in Figure 13.12. Hence, a layer of liquid is depleted of polymer. This means that the concentration of polymers in the bulk liquid is increased due to the presence of emulsion droplets. Consequently, the osmotic pressure Π_{osm} of the solution is increased. If now two droplets come close (i.e., become aggregated), part of their depletion layers overlap, and the concentration of polymer in the bulk liquid decreases. Hence, the osmotic pressure decreases. Since the system will always try to make the osmotic pressure as small as possible, there is a driving force for aggregation of the droplets. The interaction free energy is approximately given by

$$V_D \approx -2\pi r \Pi_{osm}(2\delta - h)^2, \quad 0 < h < 2\delta, \quad r \gg \delta \tag{13.16}$$

It is, in first approximation, proportional to the molar concentration of the polymer, and it will also depend on the solvent quality.

The result is that polysaccharides can cause depletion aggregation in foods, even at low concentrations; for example, 0.03% xanthan ($R_g \approx 30$ nm) may be sufficient [18]. An example is given in

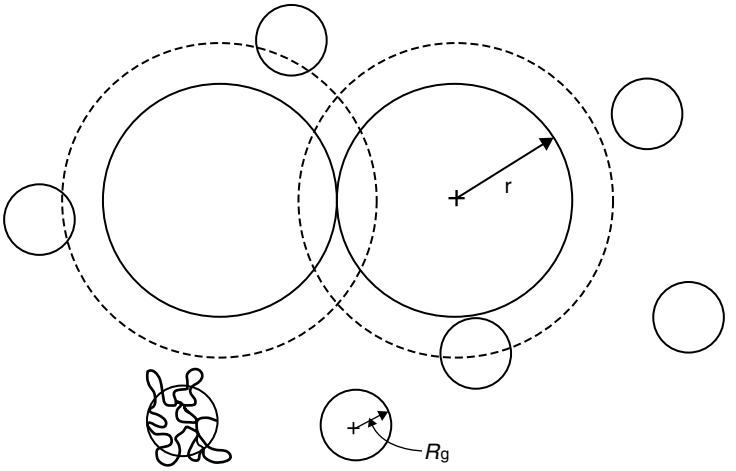

FIGURE 13.12 Schematic representation of the depletion of nonadsorbing polymer molecules (radius of gyration R_g, depicted by small circles) from the surface of colloidal particles (radius r, depicted by large circles), and of the overlap of the depletion zones (bounded by broken lines) when the particles are aggregated. (Redrawn from Walstra, P. (1993), in *Food Colloids and Polymers: Stability and Mechanical Properties* (E. Dickinson and P. Walstra, eds.), Royal Society of Chemistry, Cambridge, pp. 1–15.)

TABLE 13.4
Factors Affecting the Magnitude of Various Contributions to the Interaction Free Energy (V) Between Particles in Aqueous Systems

Variable	V_A	V_E	V_S
Particle size	+	+	(+)
Particle material	+	−	−
Adsorbed layer	(+)	+	+
pH	−	+	−[a]
Ionic strength	−	+	−[a]
Solvent quality	−	−	+

Subscripts: A = van der Waals attraction; E = electrostatic repulsion; S = steric repulsion; +, effect; −, no effect; (+), effect under some conditions.
[a] In the absence of electrical charges.

curve 4 of Figure 13.11. Substantially higher concentrations of polymer often lead to the formation of a particle gel, which implies immobilization of the particles (Sections 13.4.2 and 13.5.1).

13.3.6 Other Aspects

It should now be clear that several kinds of colloidal interactions can occur in foods and that the kind and concentration of surfactants present strongly influence these interactions. Even in the simplest cases, several variables are important (Table 13.4).

Several additional complications may be mentioned. The DLVO theory does not apply at very small distances, nor is the predicted effect of particle size obeyed. The cause may be surface roughness.

At very small distances *hydrophobic interactions* may occur, and they generally cause attraction. The effect is the result of poor solvent quality. This type of interaction has a strong temperature dependence, being very weak near 0°C and increasing with increasing temperature.

Such hydrophobic interactions may, in principle, occur if a *protein* is the surfactant. However, even in this case, the net result tends to be repulsion. This is due to a combination of steric and electrostatic repulsion, but calculation of the interaction free energy generally is not possible. If the pH is near the isoelectric point of the adsorbed protein, electrostatic repulsion may change into electrostatic attraction between negative and positive groups on the surfaces. Moreover, hydrophobic attraction can now occur and protein-covered particles generally aggregate near their isoelectric pH.

13.4 LIQUID DISPERSIONS

13.4.1 Description

Several types of liquid dispersions exist. The discussion here will be limited to suspensions (solid particles in a liquid) and to those aspects of emulsions that follow the same rules. Foods that are suspensions include skim milk (casein micelles in milk serum), fat crystals in oil, many fruit and vegetable juices (cells, cell clusters, and cell fragments in an aqueous solution), and some fabricated foods (e.g., soups). During processing (food fabrication), suspensions are also encountered, for example, starch granules in water, sugar crystals in a saturated solution, and protein aggregates in an aqueous phase.

Dispersions are subject to several kinds of instability and these are schematically illustrated in Figure 13.13. Changes in particle size and in their arrangement are distinguished. Formation of small aggregates of particles may be considered to belong to both categories. Dissolution and growth of particles depend on the concentration of the material, its solubility, and on diffusion.

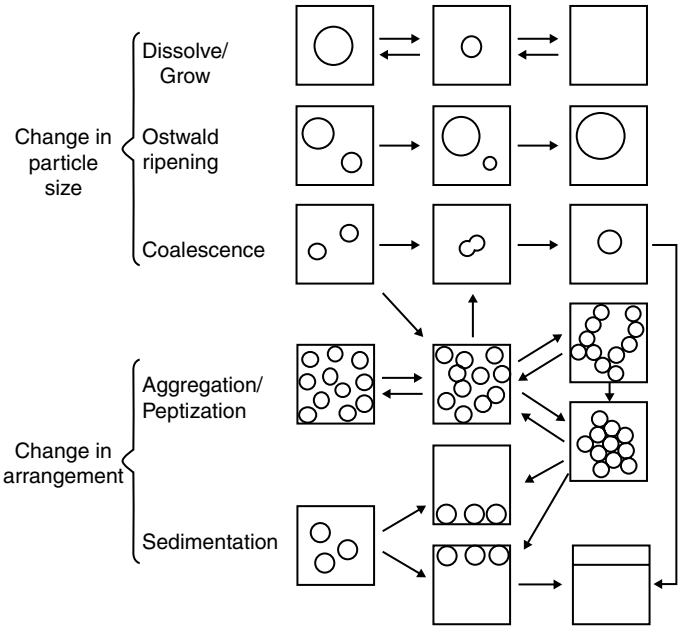

FIGURE 13.13 Illustration of the various changes in dispersity. Highly schematic.

In a supersaturated solution nucleation must occur before particles can be formed. Dissolution, nucleation, and growth will not be discussed further. Ostwald ripening is discussed in Sections 13.2.4 and 13.7.2 and coalescence in Section 13.6.4. The other changes are discussed below.

The various changes may affect each other, as is illustrated in Figure 13.13. Moreover, sedimentation is enhanced by any growth in particle size, and sedimentation will enhance the rate of aggregation if the particles tend to aggregate. Agitation of the liquid may enhance the rate of some changes, but it can also disturb sedimentation and disrupt large aggregates.

13.4.2 Sedimentation

If there is a difference in density (ρ) between the dispersed phase (subscript D) and the continuous phase (subscript C), there is a buoyancy force acting on the particles. According to Archimedes, the net force in the direction of sedimentation is for spheres given by $a\pi d^3(\rho_D - \rho_C)/6$, where a is acceleration. As the sphere accelerates it encounters a friction force, which equals, according to Stokes, $3\pi d\eta_C v$, where η_C is the viscosity of the continuous phase and v is the instantaneous velocity (with respect to the continuous phase). By putting both forces equal, the equilibrium or Stokes sedimentation velocity is obtained:

$$v_S = \frac{a(\rho_D - \rho_C)d^2}{18\eta_C} \tag{13.17}$$

If the particles show a size distribution, d^2 should be replaced by $\Sigma n_i d_i^5 / \Sigma n_i d_i^3$, where n_i is the number of particles per unit volume in class i with diameter d_i.

For gravity sedimentation $a = g = 9.81$ m s^{-2}, for centrifugal sedimentation $a = R\omega^2$, where R is the effective radius of the centrifuge and ω its rotation rate in radians per second. To give an example: if the sphere diameter is 1 μm, the density difference is 100 kg m^{-3} and the viscosity of the continuous phase is 1 mPa s (i.e., water), then the spheres would sediment under gravity at a rate of 55 nm s^{-1} or 4.7 mm per day. Sedimentation greatly depends on particle size, and spheres of 10 μm would move 47 cm in a day. Normally, viscosity decreases and sedimentation rate increases with increasing temperature. If the density difference in Equation 13.17 is negative, sedimentation is upwards, and one commonly speaks of creaming; downwards sedimentation may be called settling.

The Stokes equation is very useful to predict trends, but it is almost never truly valid. Among the many factors causing deviation from Equation 13.17 [85], the following are the most important for foods:

1. The particles are not homogeneous spheres. An anisometric particle tends to sediment more slowly, because it orients itself during sedimentation in such a way as to maximize friction (i.e., a plate-shaped particle will adopt a "horizontal" orientation). An aggregate of particles, even if spherical, sediments more slowly than a homogeneous sphere of the same size, since the interstitial liquid in the aggregate causes the effective density difference to be smaller.
2. Convection currents in the dispersion caused, for instance, by slight temperature fluctuations, may strongly disturb sedimentation of small particles (<-1 μm).
3. If the volume fraction of particles ϕ is not very small, sedimentation is hindered, roughly according to $v = v_S(1 - \phi)^8$. For $\phi = 0.1$, the sedimentation rate then is already reduced by 57%.
4. If particles aggregate, sedimentation velocity increases: the increase in d^2 is always larger than the decrease in $\Delta\rho$. Moreover, as larger aggregates sediment faster they overtake smaller ones, and thus become even larger, leading to an even greater acceleration of sedimentation rate. This may enhance sedimentation by orders of magnitude. A good

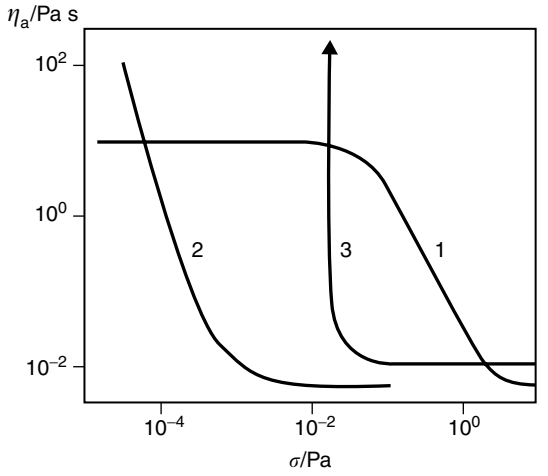

FIGURE 13.14 Schematic examples of non-Newtonian flow behavior of liquids: apparent viscosity η_a as a function of shearing stress σ. Curves (1) is typical for a polymer solution; Curve (2) for a weakly aggregating dispersion of very small particles; and Curve (3) for a system exhibiting a yield stress. (Walstra, P. (1996), in *Encyclopedia of Emulsion Technology*, Vol. 4 (P. Becher, ed.), Dekker, New York, pp. 1–62.)

example is rapid creaming in cold raw milk, where fat globules aggregate due to the presence of cryoglobulins [88].

5. An assumption implicit in Equation 13.17 is that viscosity is Newtonian, that is, independent of shear rate (or shear stress) and this is not true for many liquid foods. Figure 13.14 gives some examples of the dependence of apparent viscosity η_a on shear stress. The stress caused by a particle is given by the buoyancy force over the particle cross section, which is about $dg\Delta\rho$ for spheres under gravity. The stress is on the order of 1 mPa for many particles. This then is the stress that the particles sense during sedimentation. Viscosity should be measured at that stress (σ) (or the corresponding shear rate, given by σ/η_a), whereas most viscometers apply stresses of well over 1 Pa. Figure 13.14 shows that the apparent viscosity can differ by orders of magnitude, according to the shear stress applied.

Also shown in Figure 13.14 is an example of a liquid exhibiting a small yield stress. Below that stress the liquid will not flow. This is, however, never noticed during handling, because the yield stress is so very small (a stress of 1 Pa corresponds to a water "column" of 0.1 mm height). Nevertheless, it is often sufficient to prevent sedimentation (or creaming), as well as aggregation. Among liquid foods exhibiting a yield stress are soya milk, many fruit juices, chocolate milk, and several dressings. These aspects are discussed further in Section 13.5.2.

13.4.3 Aggregation

Particles in a liquid exhibit Brownian motion and thereby frequently encounter each other. Such encounters may lead to aggregation, defined as a state in which the particles stay close together for a much longer time than they would do in the absence of attractive colloidal interaction. The rate of aggregation is usually calculated according to Smoluchowski's theory of perikinetic aggregation [62]. The initial aggregation rate in a dilute dispersion of spheres of equal size is

$$-\frac{\mathrm{d}N}{\mathrm{d}t} = \frac{4kTN^2}{3\eta W} \tag{13.18}$$

where N is the number of particles, that is, unaggregated particles plus aggregates, per unit volume. The stability factor W was assumed to equal unity by Smoluchowski. The time needed to halve the number of particles then is

$$t_{0.5} = \frac{\pi \eta d^3}{8kT\phi} \tag{13.18a}$$

where ϕ is the particle volume fraction. This results in $d^3/10\phi$ seconds for particles in water at room temperature where d is in μm. For $d = 1$ μm and $\phi = 0.1$, this results in 1 s, implying that aggregation would be very rapid.

In most practical situations, aggregation is much slower, because W often has a large value. The magnitude of the stability factor is primarily determined by the colloidal repulsion between the particles (Section 13.3). If it is desired, for example, to increase the halving time from 1 s to 4 months, this would need a W-value of 10^7.

Direct use of Equation 13.18 to predict stability is rarely possible in food systems. There are numerous complications, and some of the more important ones are as follows: (1) it is generally impossible to predict the value of W; (2) the stability factor may change with time (an example is enzymatic hydrolysis of $-COOCH_3$ groups on pectin to $-COO^-$ groups, which can then form bridges with Ca^{2+} ions present); (3) there are other encounter mechanisms, due to streaming (agitation) or sedimentation; and (4) aggregation may take various forms, leading to coalescence (which may occur with oil droplets) or to the formation of aggregates. Nevertheless, application of aggregation theory often is possible and useful, but it is far more intricate than can be discussed here [9,83,85].

According to the nature of the interaction forces between aggregated particles (Section 13.3.3), agents can be added to cause *deaggregation*. This can be done to stabilize a food product, and also—in the laboratory—to establish the nature of the forces. It should be realized that often more than one type of force is acting. Diluting with water may cause deaggregation due to (1) lowering of osmotic pressure (if depletion interaction was the main cause of aggregation), (2) lowering ionic strength (which enhances electrostatic repulsion), or (3) enhancing solvent quality (which can increase steric repulsion). Electric forces can also be manipulated by altering pH. Bridging by divalent cations can often be undone by the addition of a chelating agent, say EDTA. Bridging by adsorbed polymers or proteins can mostly be undone by the addition of a suitable small-molecule surfactant (Section 13.2.2). Reversal of specific interactions (e.g., $-S-S-$ bridges) requires specific reagents. Also, a change in temperature can affect aggregate stability, by altering solvent quality.

If the forces between the particles in an aggregate are not very strong, deaggregation can be achieved by *shear forces*. These exert a stress $\eta \cdot \nabla v$, where ∇v is the velocity gradient (shear rate). In water, $\nabla v = 10^3\ s^{-1}$ would be needed to achieve a shear stress of 1 Pa, which does not seem very large. Nevertheless, shear stresses as occurring during agitation and flow are often sufficient to (partly) break up large aggregates.

Another aspect is that bonds may *strengthen* after aggregation. It may be better to speak of *junctions* between particles, since any such junction may represent many (up to, say, a hundred) separate bonds. Strengthening can occur by several mechanisms [83].

Aggregation of particles in liquid foods often is undesirable. It may lead to inhomogeneity of the product, for example, because aggregation strongly enhances sedimentation, or it may induce coalescence of emulsion droplets. In other cases, weak aggregation may be desirable. It may lead to the formation of a space-filling network of aggregated particles, hence to a (weak) gel. This is further discussed in Section 13.5.1.5. Consequently, the particles are immobilized and do not, or very sluggishly, sediment. Examples are cocoa particles in chocolate milk, and cells and tissue fragments in soy bean milk (see Figures 13.16 and 13.19).

13.5 SOFT SOLIDS

Many foods are "soft solids," for example, bread, margarine, peanut butter, tomato ketchup, and cheese. Another term often used is "semisolid." Both terms are ill-defined. They exclude foods that readily flow, as well as true solids, that is, foods that show at most an elastic (i.e., fully recoverable) deformation under a force applied by hand. Virtually all soft solids are composite materials, which implies that they are inhomogeneous on a mesoscopic, or even on a macroscopic, scale. The main structural classes are as follows:

Gels. These are characterized by a predominance of liquid ("solvent") and the presence of a continuous matrix of interconnected material. This space-filling network provides the solid character.

Closely packed systems. In these, deformable particles make up by far the largest volume fraction, whereby they deform each other to some extent. The interstitial material is a liquid or, in a few cases, a weak gel. Examples are vegetable purées (e.g., tomato ketchup and apple sauce), concentrated emulsions (e.g., mayonnaise), and polyhedral foams (e.g., beer foam). Concentrated starch gels consisting of highly swollen, partially gelatinized, starch granules also belong to this category; when the granules are destroyed, a highly viscous macromolecular "solution" results.

Cellular materials. Most vegetable and fruit tissues belong to this category. They are characterized by connected, fairly rigid, cell walls, enclosing a liquid-like material.

Not all soft solids fit this classification. Meat, for example, has a fibrous structure. Moreover, intermediate types occur. In this chapter we will primarily discuss gels.

13.5.1 Gels: Characterization

13.5.1.1 Structure

Types of gels may be distinguished on the basis of various criteria. For food gels, a main division is in polymer and particle networks (see Figure 13.15).

Polymer gels. The matrix consists of long, linear chain molecules, each of which is cross-linked to other molecules at various places along the chain. A subdivision can be made according to the nature of the cross-links: covalent bonds (Figure 13.15a) and physical (noncovalent) cross-links. The latter are predominant in food gels, for instance, salt bridges, microcrystalline regions (Figure 13.15b), or specific kinds of entanglements (see Sections 13.5.3.1 and 13.5.3.2). Another subdivision is that the chains between cross-links can be quite flexible, as in gelatin gels, or rather stiff, as in most polysaccharide gels.

Particle gels are illustrated in Figure 13.15c. As compared to polymer gels, most particle gel networks are much coarser (larger pores). A subdivision can be made into gels built of hard particles, such as the triacylglycerol crystals in plastic fats, and of deformable particles, such as the casein micelles in various milk gels (e.g., set yoghurt).

It may further be noted that the physical cross-links between polymer molecules, as a well as the regions of contact between particles, are better not called "bonds" but "junctions," since such a junction generally contains many individual bonds, say, 10–100. Moreover, the bonds in one junction may be of various natures (e.g., van der Waals, electrostatic, hydrophobic, and hydrogen bonds). Some proteins can also be cross-linked by covalent bonds (e.g., intermolecular –S–S– bonds).

Gelation can be *induced* in various ways, depending on the nature of the gelling material. In general, one may distinguish:

Cold-set gels. These are formed after heating to a temperature at which the network-forming material dissolves or forms a dispersion of very small particles. On subsequent cooling, a gel is formed as a result of the formation of physical cross-links. Examples are gelatin, κ-carrageenan, and mixtures of locust bean gum and xanthan, and also plastic fats. In the case of polymer gels, cooling often involves a conformational transition of the network-forming molecules, for example, in κ-carrageenan.

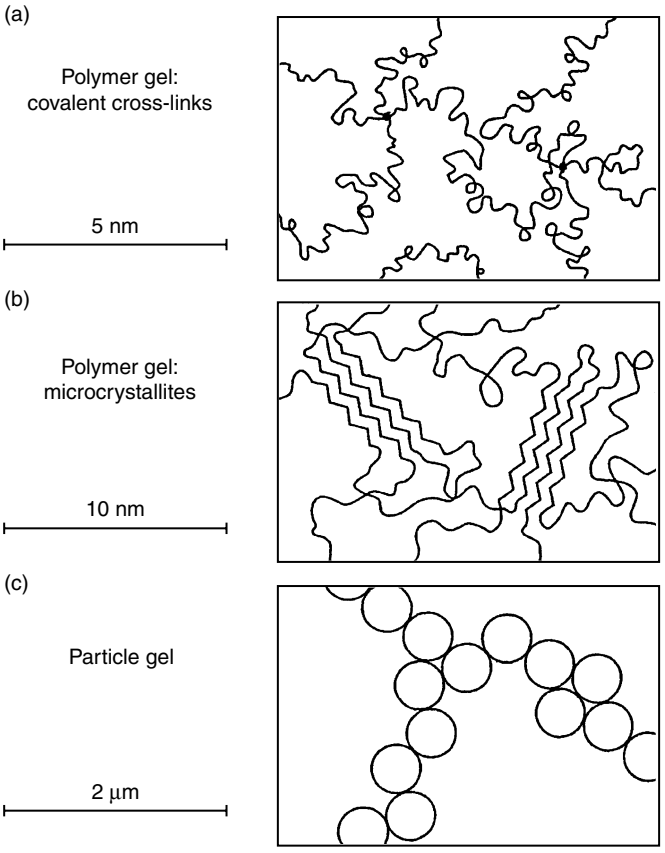

FIGURE 13.15 Highly schematic illustration of three types of gel structure. The dots in (a) denote cross-links. Note the differences in scale. (Walstra, P. (2003), *Physical Chemistry of Foods*, Marcel Dekker, New York.)

Heat-set gels. When a solution of globular proteins is heated above its denaturation temperature, a gel may be formed if the protein concentration is above a critical value c_0; generally, these gels are irreversible and considerably increase in firmness upon cooling. The magnitude of c_0 depends on the nature of the protein, the physico-chemical conditions, and the rate of heating. Examples are egg-white, soy protein isolate, whey proteins, and meat proteins. Moreover, some chemically modified polysaccharides can form reversible gels at high temperature. It mainly concerns some cellulose ethers, for example, methyl cellulose; this contains $-OCH_3$ groups, which form hydrophobic bonds at high temperature.

Some gels are formed by *changing conditions* that affect molecular or colloidal interactions, such as pH, ionic strength, specific salts (e.g., Ca^{2+} ions), or enzyme action. Examples are rennet- and acid-induced milk gels, and cold gelation by a pH change of a dispersion of thermally induced globular-protein aggregates (e.g., of β-lactoglobulin or ovalbumin).

13.5.1.2 Rheological Parameters

Most usage and eating properties of food gels are largely determined by their mechanical behavior (Section 13.5.2). For a better understanding of these aspects, we will now discuss some basic mechanical properties.

From a rheological point of view, a gel is a material that shows a predominantly *elastic* behavior over the time scale considered and has a modulus that is relatively small ($<10^7$ Pa) as compared to

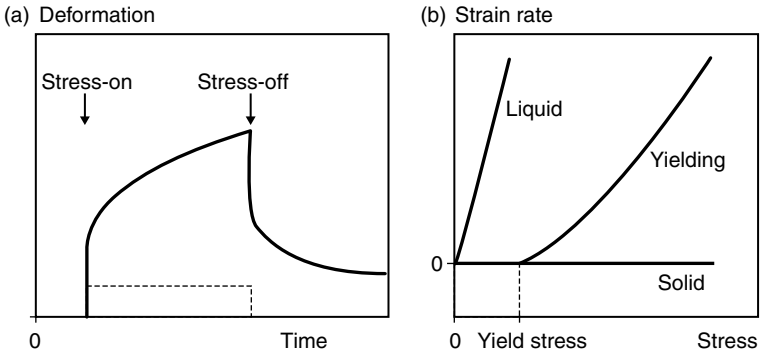

FIGURE 13.16 Viscoelasticity. (a) Example of the relation between deformation (strain) and time when a viscoelastic material is suddenly brought under a given stress, as well as after removal of the stress; the broken line is for a stress below the yield stress of the material. (b) Strain rate as a function of stress for a Newtonian liquid, a soft solid that shows yielding, and an elastic solid.

true solids. A modulus is defined as the ratio between a stress (σ/force/area) acting on a material and its ensuing relative deformation (or strain, ε); this only applies if σ/ε is independent of ε, which generally means for small deformations. Elastic behavior implies that the material instantaneously deforms under an applied stress to a strain that remains constant in time, but returns instantaneously to its original shape as soon as the stress is released (see the broken line in Figure 13.16a).

However, for many gels the deformation is not purely instantaneous: after an initial elastic deformation, the material gradually deforms further during application of the stress (see Figure 13.16a). After removal of the stress, the gel does not return to its original shape, the difference increasing with the time during which the stress was applied. The gel thus exhibits a combination of elastic and viscous (flow) behavior; it is said to behave in a *viscoelastic* manner. Gelatin and κ-carrageenan gels, at temperatures well below their gel points, show almost pure elastic behavior, whereas rennet- and acid-induced milk gels are clearly viscoelastic.

Under a large stress a gel may fracture or yield, depending on its structure and—in some gels—on the rate at which the stress is increased. *Fracture* implies that the stressed specimen breaks, mostly into several pieces. If the material contains a large proportion of solvent in wide pores, the space between the pieces may be immediately filled with solvent, as is the case when a rennet-induced milk gel is cut during cheese making. *Yielding* implies that the gel starts to flow, while remaining a coherent mass (see Figure 13.16b). Butter, margarine, and most types of jam are examples of gels that yield, while gelatin, agar, and κ-carrageenan gels fracture.

The mechanical properties of gels vary greatly. Figure 13.17a gives a hypothetical stress–strain curve, ending at the point where fracture occurs. The modulus of the material G, also called *stiffness*, is the stress divided by the strain, provided that this quotient is constant; generally, the latter is only true for very small strains, often $<1\%$. The *strength* of a material is primarily related to the stress at fracture σ_{fr}, not to the modulus. Terms like firmness, hardness, and strength are often used indiscriminately, but the sensorial attribute generally correlates with the fracture stress. Modulus and fracture stress need not be closely correlated, when comparing gels made at various concentrations (see Figure 13.17b). It is frequently observed that the addition of inert particles ("fillers") to a gelling material increases the modulus, but decreases the fracture stress [40]. Part of the explanation is that a modulus is predominantly determined by number and strength of the bonds in the gel, whereas fracture properties strongly depend on the presence of large-scale inhomogeneities.

Properties described as "shortness" and "brittleness" are closely related to the reciprocal of the strain at fracture (ε_{fr}). The latter may vary widely. For gelatin ε_{fr} equals about 3, and for some polysaccharide gels a mere 0.1. For gels as depicted in Figure 13.15a and 13.15b, ε_{fr} greatly depends on the length and stiffness of the chains between the cross-links.

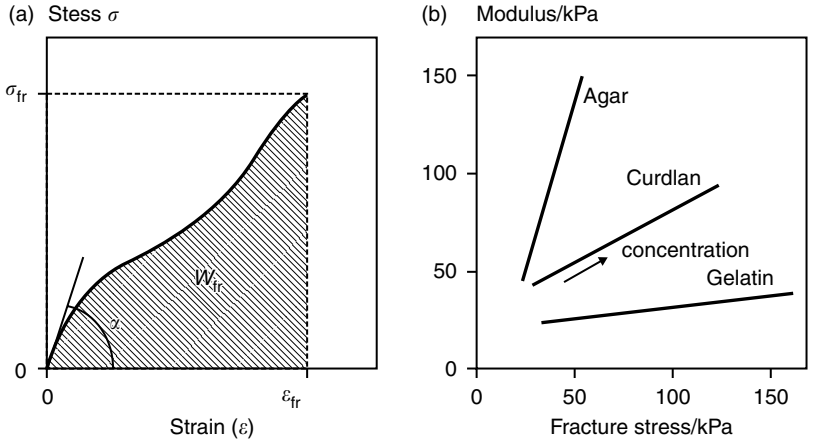

FIGURE 13.17 Large deformation. (a) Hypothetical example of the relation between stress and strain when a soft solid is deformed until it fractures; the modulus equals tan α. W_{fr} denotes the work of fracture. (b) Relation between modulus and fracture stress for gels of various materials (curdlan is a bacterial β-1,3-glucan polymer) at varying concentration. (After Kimura, H., S. Morikata and M. Misaki (1973), *J. Food Sci.* **38**: 668–670.)

Another parameter is "toughness," which is related to the work of fracture W_{fr}. This derives from the area under the curve in Figure 13.17a, and is expressed in J m^{-3}.

13.5.1.3 The Modulus

At small deformations gels can be characterized by a modulus. A very general expression will be given for the modulus, based on a simplified model of a gel. In this model, the gel is built of strands that are mutually cross-linked. A strand can consist of a polymer chain or a chain of aggregated particles. When a force is applied to such a chain, this will result in a reaction force in the chain. This force is proportional to the deformation Δx times the derivative of the interaction force f with respect to the distance x between the cross-links, df/dx. When multiplying both sides of the equation by the number N of stress-carrying strands per unit cross section, the following expression is obtained

$$\sigma = -N\frac{df}{dx}\Delta x \tag{13.19}$$

The local change in distance can be recalculated to a macroscopic strain ε by dividing Δx by a characteristic length C, which is determined by the geometry of the network. (The calculation of C is intricate and is not given here.) Since f can generally be expressed as the derivative of the (Gibbs) free energy F with respect to distance x, we obtain

$$\sigma = CN\frac{d^2F}{dx^2}\varepsilon \tag{13.20}$$

Since $G = \sigma/\varepsilon$ and $dF = dH - TdS$, where H is enthalpy and S entropy, the following expression for the modulus results

$$G = CN\frac{d^2F}{dx^2} = CN\frac{d(dH - TdS)}{dx^2} \tag{13.21}$$

13.5.1.4 Polymer Gels

Deformation of a gel with long and flexible polymer chains between cross-links leads primarily to a change in conformation of these chains, implying that the entropy of the network decreases. This means that the enthalpy term in Equation 13.21 may be neglected. For gels with stiff polymer chains between cross-links, deformation also implies a change in enthalpy, since chemical bonds in the chains are being bent or stretched. Most polysaccharide gels are in this category, and in some of these the entropy change may even be neglected.

It should further be remarked that in the derivation of [21] it is implicitly assumed that the properties of all the strands are identical. This is generally not the case, especially because the cross-links often have the form of junctions, where number and strength of the bonds in a junction may vary substantially. However, in the simple case depicted in Figure 13.15a—chemical cross-links and long flexible chains—Equation 13.21 can be reduced to a very simple expression for the modulus

$$G = \nu kT \tag{13.22}$$

where ν is the number of chains between cross-links per unit volume. This equation is well obeyed for very small deformations, provided that the value of ν does not change with temperature or other variables (pH, ionic strength, or solvent quality during gelation).

By the application of percolation theories, simple scaling laws for the modulus of polymer gels with the concentration of gel-forming material c have been derived, for instance [65].

$$G \propto (c - c_0)^n \tag{13.23}$$

where the exponent n varies, for the most part between 2 and 4, depending on the structure of the network. There is a minimum concentration c_0 for gel formation, but a physical explanation for its value is not provided. These values depend on the nature of the gel-forming material and on the physico-chemical conditions during gelation. Often, relation [23] can be fitted well to experimental results.

13.5.1.5 Particle Gels

These gels can form due to aggregation of particles that are made to attract each other, for example, by a change in pH, ionic strength, or solvent quality. The structure of the aggregates formed is generally of a *fractal* nature [92]. When "attractive" particles encounter each other at random, they form small aggregates (at first doublets), and as these encounter other aggregates, larger ones result. This is called cluster–cluster aggregation. A simple relation tends to develop between the (average) number of particles in an aggregate N_p and the radius R of the aggregate

$$N_\mathrm{p} = \left(\frac{R}{r}\right)^D \tag{13.24}$$

where r is the radius of the primary particles. D is a constant <3, which is called the *fractal dimensionality*. Because it is smaller than 3, larger aggregates are more tenuous (rarefied) than smaller ones. The average volume fraction of particles in an aggregate is given by

$$\phi_\mathrm{agg} = \frac{N_\mathrm{p}}{N_\mathrm{m}} = \frac{(R/r)^D}{(R/r)^3} = (R/r)^{D-3} \tag{13.25}$$

where N_m is the number of primary particles that a sphere of radius R would obtain on close packing. Since $D < 3$, ϕ_agg decreases as R increases, until it equals the volume fraction of primary particles

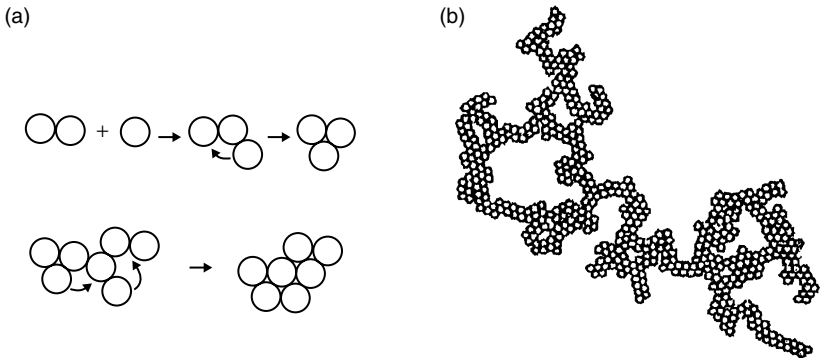

FIGURE 13.18 Sort-term rearrangement in fractal aggregates. (a) Examples of particles rolling over each other so that a higher coordination number is attained. (b) Example of a fractal aggregate in two dimensions where some short-term arrangement has occurred. (Walstra, P. (2003), *Physical Chemistry of Foods*, Marcel Dekker, New York.)

in the system ϕ. In principle, all particles will then be included in aggregates, which fill the system. Bonds between aggregates have formed, resulting in a space-filling network (i.e., a gel). The critical radius of the aggregates at the gel point is given by

$$R_g = r\phi^{1/(D-3)} \tag{13.26}$$

This mechanism implies that a gel will be formed at any value of ϕ, however small. However, at very small ϕ, the gel will be too weak to be noticed and it will readily be broken up by weak agitation. Moreover, sedimentation of aggregates may occur before a gel can form. This disturbance will happen more readily when ϕ is smaller; hence, there will be a critical concentration for gelation ϕ_0.

An often occurring complication is that soon after small aggregates have formed, particle rearrangement occurs, as illustrated in Figure 13.18. Particles may roll over each other until they have formed bonds with more than one other particle. The fractal nature of the aggregates remains, but instead of r, a larger "effective" radius r_e has to be used in the equations. Moreover, it will imply a higher value of ϕ_0, due to faster sedimentation of small aggregates.

The theory of fractal aggregation allows the derivation of scaling laws for rheological parameters of the gel formed. It is assumed that the modulus of the gel derives exclusively from a change in enthalpy upon deformation. It is further taken into account that fractal structures are (on average) self-similar. This implies that the number of bonds (or junctions) between adjacent aggregates is independent of their radius. The contact area between aggregates in the final gel will scale with R_g^2. This implies that the number of bonds between the aggregates per unit cross section of the gel scales with R_g^{-2}, hence with the number of stress-carrying strands N in Equation 13.21. It causes the modulus to scale with ϕ as

$$G \propto C\frac{d^2H}{dx^2}\phi^{2/(3-D)} \tag{13.27}$$

Often, $D \approx 2.2$, causing G to be proportional to about $\phi^{2.5}$. Since also C may depend on ϕ, both weaker and stronger dependencies of G on ϕ may be observed [46].

In conclusion, although particle gels seem to be rather disordered structures, simple scaling laws to describe various properties often hold (see also Equation 13.29).

13.5.2 Functional Properties

Food technologists make gels for a purpose, often to obtain a certain consistency or to provide physical stability. The properties desired and the means of achieving them are summarized in Tables 13.5 and 13.6.

Consistency has already been briefly discussed, but the message of Table 13.5 is an important one: according to the purpose in mind, the rheological measurements must be of a relevant type and conducted at the relevant time scale or strain rate. This need not be difficult. For instance, to evaluate stand-up, which is the propensity of a piece of gel (say, a pudding) to keep its shape under its own weight, measurement of a modulus makes no sense. The proper experiment is simply watching the piece, and possibly measuring the height of a specimen that will just start yielding. To ensure stand-up, the yield stress must be greater than $g \times \rho \times H$, where H is specimen height. For a piece 10 cm tall, this would be about $9.8 \times 10^3 \times 0.1 \approx 10^3$ Pa. It should further be realized that the yield stress often is smaller when the time scale is longer.

Very *weak gels* were briefly discussed in Section 13.4.3. In daily life, such a system appears to be liquid, as it will readily flow out of a bottle if its yield stress is <10 Pa or so; nevertheless, it has elastic properties at extremely small stresses. This small yield stress may be sufficient to prevent sedimentation. A good example is soya milk (Figure 13.19). Soya milk contains small particles, consisting of cell fragments and organelles. These particles aggregate, forming a weak reversible

TABLE 13.5
Consistency of Gels: Desired Mechanical Characteristics of Gels Made for a Given Purpose

Property Desired	Relevant Parameters	Relevant Conditions
Stand-up	Yield stress	Time scale
Firmness	Fracture stress or yield stress	Time scale, strain
Shaping[a]	Yield stress + restoration time	Several
Handling, slicing	Fracture stress, work of fracture	Strain rate
Eating properties	Yield and fracture properties; stiffness	Strain rate
Strength (e.g., of film)	Fracture properties	Stress, time scale

[a] After making the gel.

TABLE 13.6
Gel Properties Needed to Provide Physical Stability

Prevent or Impede	Gel Property Needed
Motion of particles	
Sedimentation	High viscosity or significant yield stress + short restoration time
Aggregation	High viscosity or significant yield stress
Local volume changes	
Ostwald ripening	Very high yield stress
Motion of *solvent*	
Leakage	Small permeability + significant yield stress
Convection	High viscosity or significant yield stress
Motion of solute	
Diffusion	Very small permeability, high solvent viscosity

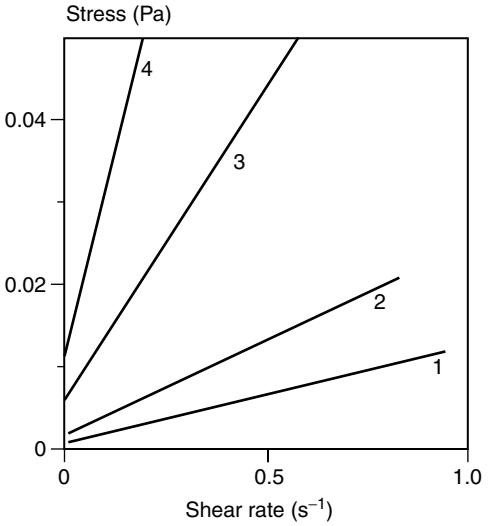

FIGURE 13.19 Flow curves (shear stress vs. shear rate) of soya milk. The yield stresses are given by the y intercepts of the curves. The soya milks were made of dehulled (Curves 1 and 2) or whole beans (3 and 4), soaking the beans overnight at room temperature (1 and 3), or 4 h at 60°C (2 and 4). (After Oguntunde, A.O., P. Walstra and T. van Vliet (1989), in *Trends in Food Biotechnology* (Ang How Ghee, ed.), Proceedings of the 7th World Congress Food Science Technology, 1987, pp. 307–308.)

gel. If processing conditions are appropriate, the yield stress is sufficient to prevent these particles, and even larger particles, from sedimenting. Also, some mixtures of polysaccharides, for example, solutions of xanthan gum and locust bean gum, even if very dilute, can exhibit a small yield stress (Figure 13.14, curve 3). This yield stress can prevent sedimentation of any particles present [41].

Permeability. Sometimes, it is desired to arrest the motion of liquid, in which a case the permeability of the gel is an essential parameter. According to Darcy's law, the superficial velocity v of a liquid through a porous matrix is

$$v \equiv \frac{Q}{A} = \frac{B}{\eta}\frac{\Delta p}{x} \tag{13.28}$$

where Q is the volume flow rate ($m^3\ s^{-1}$) through a cross-sectional area A and Δp is the pressure difference over distance x. The permeability B (in m^2) is a material constant that varies greatly among gels. A particle gel like renneted milk (built of paracasein micelles) has a permeability on the order of 10^{-12} m^2, whereas a polymer gel (e.g., gelatin), typically would have a B of 10^{-17}. In the latter case, leakage of liquid from the gel would be negligibly slow.

For fractal particle gels a simple scaling law for the permeability can be derived

$$B = \frac{r_e^2}{K}\phi^{2/(D-3)} \tag{13.29}$$

where K is a proportionality constant, often between 50 and 100. The exponent of ϕ is very well obeyed [92].

Transport of a solute through the liquid in a gel has to occur by *diffusion*, because convection generally is not possible. The diffusion coefficient D is not greatly different from that in solution, at least for small molecules in a not very concentrated gel. The Stokes relation for diffusivity $D = kT/6\pi\eta r$, where r is molecule radius, cannot be applied. The macroscopic viscosity of the system is irrelevant, since it concerns here the viscosity as sensed by the diffusing molecules, which would

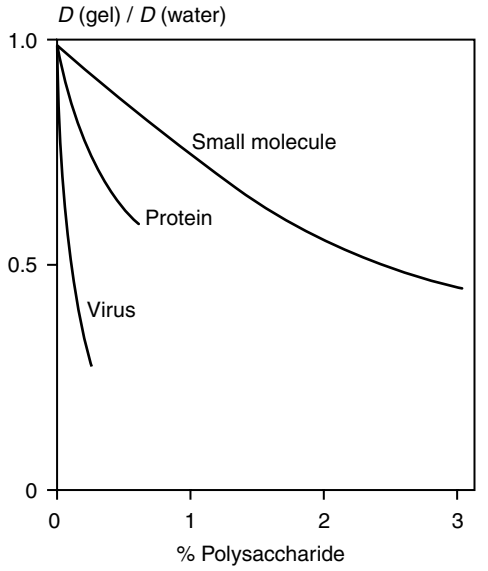

FIGURE 13.20 Diffusion of solutes in polysaccharide gels of various concentrations. D is diffusion coefficient. (Highly schematic examples after Muhr, A.H. and J.M.V. Blanshard (1982), *Polymer* **23**(suppl.): 1012–1026.)

be the viscosity of the solvent. On the other hand, the solute has to diffuse around the strands of the gel matrix, and the hindrance will be greater for larger molecules and smaller pores between strands in the gel. These aspects are illustrated in Figure 13.20.

Swelling and syneresis are additional properties of gels. Syneresis refers to expulsion of liquid from the gel, and it is the opposite of swelling. There are no general rules governing their occurrence. In a polymer gel, lowering of solvent quality (e.g., by changing temperature), adding salt (in the case of polyelectrolytes), or increasing the number of cross-links or junctions, may cause syneresis. However, because both the pressure difference in Equation 13.28 and the value of B are usually very small, syneresis (or swelling) tends to be very slow. In particle gels, syneresis may occur much faster, due to the far greater permeability. It is well known that renneted milk is prone to syneresis, an essential step in cheese making. The combination of variables influencing syneresis is intricate [75].

13.5.3 Some Food Gels

The fairly theoretical points discussed above will now be illustrated by discussing some food gels.

13.5.3.1 Polysaccharides [48]

Despite the wide range of polysaccharide types (see Chapter 3), some general rules governing their gel properties exist. Most polysaccharide chains are fairly stiff, one of the causes being that several bulky side groups may be present on the "backbone" chain. Generally, appreciable bending of a chain segment can occur only if its length exceeds about 10 monomers (monosaccharide residues). This characteristic causes polysaccharides to produce highly viscous solutions; for example, 0.1% xanthan will increase the viscosity of water by at least a factor of 10. Some polysaccharides can form gels. Broadly speaking, the gel cross-links are junctions, each containing a great number of (weak) bonds, and jointly involving an appreciable portion of the material. This means that the strands between cross-links are not very long. Combined with chain stiffness, this leads to rather short (or even brittle)

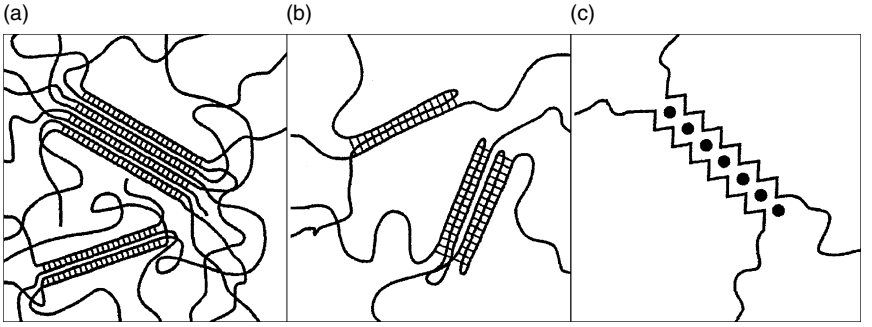

FIGURE 13.21 Various types of junctions in polymer gels. (a) Stacked double helices, for example, in carrageenans. (b) Triple helices in gelatin. (c) "*Egg-box junction*", for example, in alginate; the dots denote Ca ions. Highly schematic; helices indicated by hatching.

gels. Actually, they are intermediate between entropic and enthalpic gels (cf. Equation 13.21). Of course, there is considerable variation among polysaccharides in this regard.

Cross-links among polysaccharide molecules can be any of the following three types.

Microcrystallites (Type 1). The simplest type, local stacking of stretched chain segments, is depicted in Figure 13.15b. This type is not common in gelling polysaccharides (although native cellulose is an example of an almost completely crystallized linear polymer.) Amylose cannot form linear chains, but stacks of single amylose helices can presumably form microcrystalline regions in solutions and, if the amylose concentration is high enough, gelation will occur. With amylopectin, similar behavior is observed. These phenomena are involved in "retrogradation" of gelatinized starch. Microcrystallites can also be formed of other structural elements (see below).

Double helices (Type 2). Several polysaccharides (e.g., carrageenans, agar, and gellan) can form double helices below a sharply defined temperature, which depends on conditions. Each helix generally involves two molecules, but they can only be formed in so-called nonhairy regions of the polymer (i.e., regions that are devoid of bulky side groups). Double helices would thus be cross-links leading to gelation. However, helix formation tends to be very rapid (milliseconds), whereas gelation takes far longer (several seconds). The double helices do form microcrystallites (see Figure 13.21a), at least in κ-carrageenan, which presumably stabilizes them. As soon as the helices "melt," so does the gel.

Egg-box junctions (Type 3). These occur with some charged polysaccharides, such as alginate, when divalent cations are present (Figure 13.21c). Alginate has negative charges, often spaced at regular distances, allowing divalent cations, such as Ca^{2+}, to establish bridges between two parallel polymer molecules. In this way, fairly rigid junctions are formed. It is likely that the junctions further arrange themselves in microcrystalline regions. The junctions do not readily "melt" unless the temperature is near 100°C.

Many factors may affect gelation and gel properties of polysaccharides. These include molecular structure, molar mass (Figure 13.22b), concentration (Figure 13.22a and 13.22b), temperature (Figure 13.23b), solvent quality, and for polyelectrolytes, pH, and ionic strength (Figure 13.23b).

13.5.3.2 Gelatin [11,37]

Of all the food gels, gelatin is closest to an ideal entropic gel. The flexible molecular strands between cross-links are long and this causes the gel to be very extensible. It is also predominantly elastic, because the cross-links are fairly permanent (at least at low temperature). However, the relation given for the modulus in Equation 13.22 is not well obeyed. The modulus roughly increases with the square of the concentration (Figure 13.22a) and the temperature dependence is very different from the

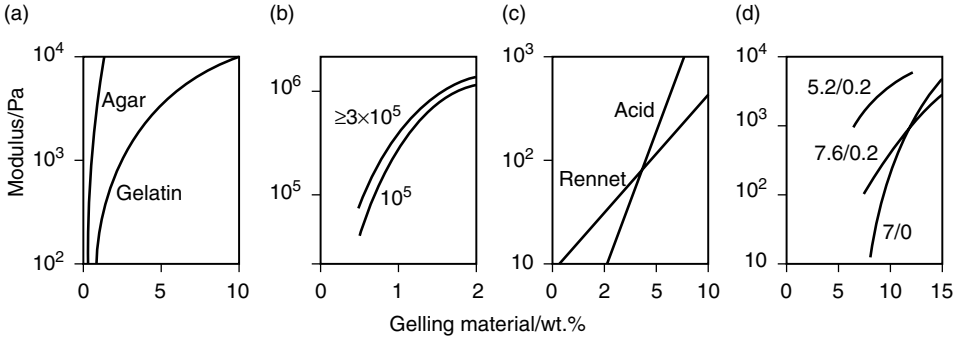

FIGURE 13.22 Effect of concentration of gel-forming material on the shear modulus of the gels. (a) Agar and gelatin. (b) κ-Carrageenan of two molar masses (indicated) in 0.1% KCl. (c) Casein gels made by slow acidification or by renneting. (d) Heat-set gels of soya protein isolate; figures near the curves indicate pH/added NaCl (molar).

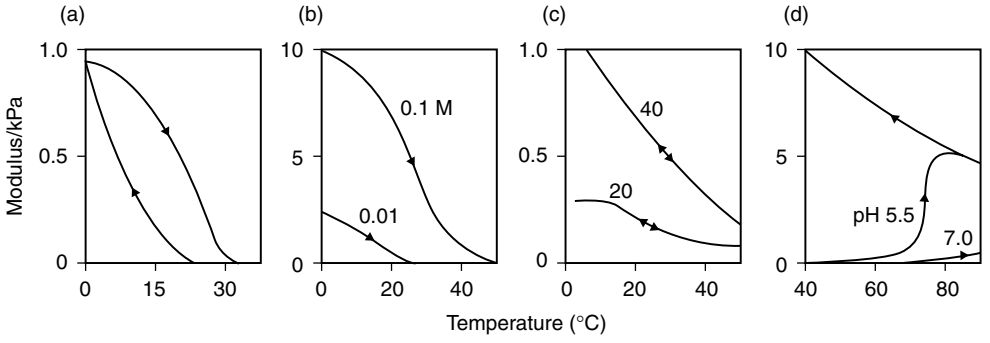

FIGURE 13.23 Effect of measurement temperature on the shear modulus of various gels. Arrows indicate the temperature sequence. (a) Gelatin (2.5%). (b) κ-Carrageenan (1%) for two concentrations of $CaCl_2$ (indicated). (c) Acid casein gels (2.5%) made and aged at two temperatures (indicated). (d) β-Lactoglobulin (10%) at two pH values (indicated). The results will also depend on heating or cooling rate.

prediction (Figure 13.23a). These discrepancies stem from the mechanism of cross-linking. Despite the severe treatment of the collagen during preparation of gelatin, the molecules may retain much of their length and produce highly viscous aqueous solutions. Upon cooling, the molecules tend to form triple helices, like the proline helices in collagen. This applies to only part of the gelatin and the helical regions are relatively short. A gelatin molecule cannot form an intermolecular double helix, as several polysaccharides can do. This is because peptide bonds cannot rotate over the full 360°, implying that helix formation at one place will cause twisting of other parts of the molecules, which would soon stop due to steric hindrance. Presumably, a gelatin molecule sharply bends at a so-called β-turn, and then forms a short double helix. Subsequently, a third strand may wind around this helix, thereby completing it. If the third strand is part of another molecule, a cross-link is formed (see Figure 13.21b). Upon an increase of temperature, triple helices will "melt," whereby the modulus will decrease.

Actually, the gelation mechanism must be more complicated. As shown in Figure 13.23a, there is considerable hysteresis between cooling and heating curves. Also, when cooling a gelatin solution to a temperature below, say, 25°C, the modulus may keep increasing for days. This goes along with an increase in helical material, up to about 30% of the gelatin, but some structural rearrangement will also occur. It is still a matter of debate to what extent stacking of triple helices occurs.

It may finally be noticed that the temperature dependence of the gel state, which is virtually unique for gelatin, offers several possibilities for food manufacture.

13.5.3.3 Caseinate Gels [77,84,92]

Milk contains casein micelles, proteinaceous aggregates of about 120 nm average diameter, each containing some 10^4 casein molecules (see Chapter 15). The micelles can be made to aggregate, by lowering the pH to about 4.6 (thereby decreasing electric repulsion), or by adding a proteolytic enzyme that removes the parts of κ-casein molecules that protrude into the solvent (thereby decreasing steric repulsion). Fractal gels are formed, with a fractal dimensionality of about 2.3. The permeability, which is about 2×10^{-13} m^2 for average casein concentration (c), is strongly dependent on the latter, being about proportional to c^{-3}. For casein gels a linear relation exists between log modulus and log casein concentration (Figure 13.22c), in accordance with their fractal nature (Equation 13.27). The different slopes imply a difference in structure. The initially tortuous strands formed during rennet gel formation, become straightened soon afterwards, whereas the strands in acid gels remain tortuous [47].

The building blocks of the gel, that is, the casein micelles, are themselves deformable, and the junctions between them are flexible. Thus, the gel is rather weak and soft. For acid casein gels, fracture stress is about 100 Pa and the fracture strain is about 1.1; for rennet gels, these values are about 10 Pa and 3, respectively. The acid gel is thus shorter. These results apply for slow deformation, say over 15 min; at faster deformation, the fracture stress is much greater. Applying a stress of slightly over 10 Pa to a rennet gel will cause flow (there is no detectable yield stress), and after considerable time fracture will occur. Applying a stress of 100 Pa leads to fracture within 10 s. Similar behavior is found for some other types of particle gels, but it is by no means universal.

All these values depend on conditions applied, especially temperature. It is seen that the modulus of a casein gel is larger at lower temperature (Figure 13.23c). This may appear strange, since hydrophobic bonds between casein molecules are considered to play a major part in keeping the gel together, and these bonds decrease in strength with decreasing temperature. Presumably, a decrease in hydrophobic bond strength (low T) leads to swelling of the micelles; hence, to a larger contact area between adjoining micelles; hence, to a greater number of bonds per junction. Conversely, a higher temperature of gel formation leads to a larger modulus (at least for acid gels; Figure 13.23c), and this is due to a somewhat different network geometry, not to a difference in type of bonds.

At temperatures above about 20°C, rennet gels show syneresis. Syneresis goes along with rearrangement of the network of particles, which implies that some deaggregation occurs. In a region where no liquid can be expelled (i.e., internally in the gel), rearrangement occurs as well, leading to both denser and less dense regions. This is called microsyneresis; it results in an increase in permeability and causes the straightening of the network strands mentioned above.

13.5.3.4 Globular Proteins Gels [12,63,64]

Many well-soluble globular proteins form a gel upon heating, if the protein concentration is above a critical value c_0 (Figure 13.22d). These heat-set gels form only if at least part of the protein has been heat-denatured and does not return to the native state upon cooling (cf. Figure 13.23d). Gel formation is a relatively slow process, taking at least several minutes. It may take far longer to reach the maximum stiffness. Gel formation involves a number of consecutive reactions: (1) protein molecules become denatured, (2) denatured molecules aggregate to roughly spherical or to elongated particles, and (3) these particles form a space-filling network. These reactions partly proceed in parallel.

Details of gel formation and of the gel properties obtained vary widely among proteins, due to their variation in molecular structure and conformational stability. A further complication is that heat-set gels are usually made up of protein mixtures, such as whey protein or soy protein isolates. Bonds involved in gel formation include –S–S– linkages, electrostatic, van der Waals, and hydrophobic interactions, and H-bonds as part of intermolecular junctions between β-strands.

The structure, and thereby the rheological properties, also vary greatly with pH, ionic strength, salt composition, and rate of heating. In general, two types of gel structure can be distinguished by microscopy, namely, fine-stranded and coarse-stranded networks. The former gels are clear (transparent) and consist of relatively thin strands (diameter generally 10–50 nm) that are branched to form a network. They typically form on heating at a pH far from the isoelectric point at low ionic strength. Coarse-stranded or particle gels (some have a fractal network structure) are turbid and are generally built of roughly spherical particles of 0.1–1 μm. These gels generally form by heating at a pH close to the isoelectric point and/or high ionic strength. Coarse-stranded gels tend to be stiffer than fine-stranded ones. In both types of gel, the tortuosity of the strands may vary considerably, resulting in widely varying fracture strains. Moreover, heat-set protein gels vary substantially in permeability, and thereby in their propensity to lose solvent under pressures acting during further processing or handling.

An alternative process to make gels of globular proteins is by heating a solution at a pH where the molecules denature and form small aggregates, but do not form a gel. After cooling, the pH is brought to a value close to the isoelectric point, and a gel forms; this is known as cold gelation.

Structure formation during extrusion is comparable to heat setting of globular proteins. An important example involves protein-rich products from soya [38].

13.5.3.5 Mixed Gels

It will now be clear that the structure and the properties of gels can vary greatly. The modulus of a 1% gel can vary by almost five orders of magnitude and the strain at fracture by a factor of 100. Nearly every system exhibits specific relations that are often poorly understood.

The situation becomes even more complicated when mixed gels are considered. Relatively simple are gels filled with particles (e.g., emulsion droplets), which gels may have greatly altered properties as compared to unfilled ones [10,40]. Often, a mixture of polysaccharides is used. Weak attraction between the polymers at gel-forming conditions, may cause gelation, even if both polymers are non-gelling [10,49]. For instance, dilute xanthan or locust bean gum solutions do not show an appreciable yield stress, while dilute mixtures can do: after heating and subsequent cooling, mixed junctions are formed. Another example is the addition of, say, 0.03% κ-carrageenan to milk, which results in the formation of a weak gel. This is applied (e.g., in chocolate milk to prevent sedimentation of cocoa particles).

Phase separation may occur due to thermodynamic incompatibility of two polymers. This is a very common phenomenon, unless the polymer concentrations are quite low. For instance, mixed solutions of a highly soluble polysaccharide and a protein can separate into two phases, one rich in protein and the other rich in polysaccharide [67,68]. This can happen, for instance, in mixtures of gelatin and the nongelling polysaccharide dextran. Phase separation occurs directly after mixing at a temperature above the gel point of gelatin; if the gelatin solution is the continuous phase after phase separation, the system becomes frozen after cooling.

Strong mutual attraction between polymers may lead to the formation of a complex coacervate. This implies separation into a phase containing a high concentration of both, leaving another phase that is depleted of polymers. It can occur if the polymers have opposite charge, for instance, between β-lactoglobulin and gum arabic at pH 2.5–4.5 and low ionic strength.

Complete foods are still more complicated than the systems so far discussed [30]. Nevertheless, knowledge of the principles provided can be of great help in understanding food behavior and for designing experiments to study it.

13.5.4 Mouthfeel of Foods [32,58,74]

The eating characteristics of foods form an essential quality attribute. Fabricated foods are often specially designed to optimize these characteristics, which include flavor, texture, and appearance.

Here we will focus on texture as perceived in the mouth, which primarily involves consistency and physical inhomogeneity. Actually, the mouth can be considered a processing unit in which the food is worked, broken down and transported to the oesophagus in a manner depending on its mechanical properties. Moreover, the mouth and the connected nasal cavity contain several sense organs, which are used in the evaluation of eating characteristics of the food.

The manner in which liquids and soft solids are processed in the mouth differs from that of hard solids. Liquids are just transported to the oesophagus, mainly by use of the tongue, while the working of hard solid foods involves a few separate stages. In general, we can distinguish (1) ingestion/biting; (2) chewing and moistening, including bolus formation; and (3) swallowing of the bolus and clearance of the mouth. In each stage the food is deformed in different ways at various rates, while it is mixed with saliva. The working of soft solids also involves a pressing and shearing action between the tongue and the palate. During this processing the consumer already starts to evaluate eating characteristics, including several texture attributes, such as thickness, roughness/smoothness, mealiness, and so forth. Several of such characteristics are multicomponent attributes, as they consist of some subattributes that must all be present to a certain extent. "Creaminess," for example, involves thickness and smoothness, while roughness must be absent. (Probably, flavor can also contribute to the impression of creaminess).

The working in the mouth is best known for liquids and hard solids, although still to a limited extent; nevertheless, some general rules have been established.

The oral evaluation of a liquid depends on its flow characteristics, best expressed as the viscosity as a function of shear rate. As a rule of thumb, for low-viscosity liquids (below 0.1 Pa s) the sensory evaluation of viscosity corresponds with instrumental evaluation at a shear stress of about 10 Pa. For products with a high viscosity (above 10 Pa s), sensory evaluation corresponds with instrumental evaluation of stress at a constant shear rate of 10–20 s^{-1}; the evaluation in the mouth will often involve a smearing action between the tongue and the palate as well. For intermediate viscosities, there is a gradual transition of the rheological parameters that are determinant. "Thickness" is often considered as a single sensorial characteristic; however, besides (apparent) viscosity, other rheological properties are probably involved. This is certainly so for soft solids like apple sauce or tomato ketchup.

For low-viscosity liquids, such as water and milk, the flow in the mouth may locally be turbulent. For all liquid and many soft solid foods, the flow rate in the mouth will vary from place to place, and the food is mixed with (diluted by) saliva. Tomato ketchup, for instance, and many products based on hydrocolloid solutions, become thinner during flow. The thinning effect is perceived in the mouth and appears to be related to the texture attribute "sliminess"; however, even this simple attribute can probably not be ascribed to one rheological property [74]. For foods that have a yield stress below which they will not flow, as is the case for many soft solids, the perceived thickness will also depend on the magnitude of this yield stress. Finally, the pressing and shearing action between the tongue and the palate will induce elongational besides shear flow, and for several, especially polymeric, materials, the elongational viscosity is substantially higher than the "common" simple-shear viscosity.

The effect of temperature on the perception of viscosity and yield stress-related texture attributes is probably relatively small. However, its effect may be much larger when a phase change occurs between the initial temperature of the food and mouth temperature (e.g., melting of fat crystals or of a gelatin gel). Changes can also occur due to enzyme action. Although the residence time for many products in the mouth is only of the order of seconds, significant starch degradation may occur during this period, depending, for instance, on the extent of mixing of food and saliva.

After swallowing, a coating of food remnants is generally left on the tongue and other parts of the oral cavity. The extent of coating depends on the adhesive and cohesive properties of the food as such, and of the particulate matter remaining after processing in the mouth. The coating will markedly affect the "after-swallowing" sensory impression. For instance, binding of (macro)molecules to the mucus layer covering the oral surfaces appears to correlate with an increase of the sensory attribute astringency or roughness.

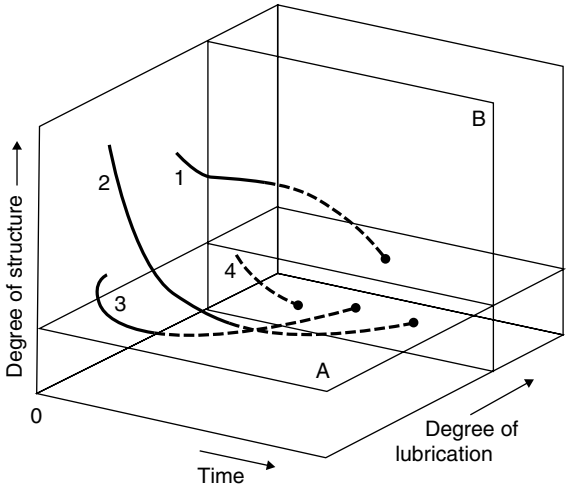

FIGURE 13.24 Schematic representation of break-down trajectories in the mouth of some foods after ingestion (at time = 0). The two main processes considered are (a) structure break-down, which is considered to be sufficient below the horizontal plane A; and (b) lubrication, which would be sufficient beyond the vertical plane B. The processed food (bolus) must fulfill both criteria before it can be swallowed. Examples are (1) tender and juicy meat; (2) tough and dry meat; (3) dry sponge cake (note that the degree of lubrication at first decreases, then increases); and (4) a thick liquid, for example, stirred yoghurt. (The lines are broken when they are below A and/or beyond B.) The points of swallowing are indicated by dots. (Modified after Hutchings, J.B. and P.J. Lillford (1988), *J. Texture Studies* **19**: 103–115.)

As mentioned above, three stages can be distinguished in the processing of a solid food in the mouth. The biting action can to a great extent be imitated by uniaxial compression between two wedges [78] at a suitable compression rate. The large-deformation properties of many food products depend on deformation rate. The biting velocity generally is between 2 and 6 cm s^{-1}. The resistance to biting and the maximum biting force required to fracture the material are related to sensory firmness and hardness, but other properties are also involved. After biting, the food will further be broken down by a grinding and shearing action between the molars. The tongue plays an important role in transporting the food particles to the molars, and in deciding which particles are sufficiently broken down and moistened to be swallowed; the speed and the relative importance of these processes depend on the type of food (see Figure 13.24). The food is then transported to the back of the oral cavity, where it forms a bolus, which is swallowed after some time. Clearance of the oral cavity can be due to mechanical action of the tongue, slow dispersion or dissolution of food remnants in saliva, and enzymatic breakdown.

13.6 EMULSIONS

13.6.1 Description

Emulsions are dispersions of one liquid into another. The most important variables determining emulsion properties are as follows:

1. *Type, that is, o/w or w/o*. This determines, among other things, with what liquid the emulsion can be diluted (Section 13.1.2). Many foods are o/w emulsions, examples being milk and milk products, sauces, dressings, and soups. Foods that are true w/o emulsions hardly exist. Butter and margarine contain aqueous drops, but these are embedded in a plastic fat;

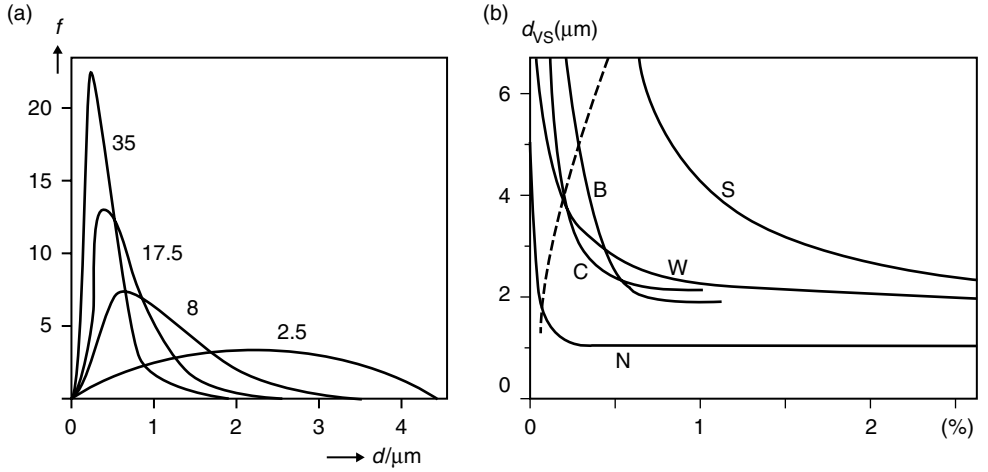

FIGURE 13.25 Emulsification: effects of various conditions on the resulting droplet size. (a) Effect of homogenization pressure (indicated near the curves in MPa) on the volume frequency distribution in percentage of the oil per 0.1 μm class width vs. droplet diameter d; 3.5% oil in skim milk. (b) Effect of emulsifier concentration (% w/w) on volume/surface average droplet diameter d_{vs} for various emulsifiers. B, blood protein; C, sodium caseinate; N, nonionic small-molecule surfactant; S, soya protein; W, whey protein (Walstra, P. (2003), *Physical Chemistry of Foods*, Marcel Dekker, New York.). Approximate results for 20% oil and moderate emulsification intensity.

melting of the crystalline part of the fat produces a w/o emulsion that immediately separates into an oily layer on top of an aqueous layer. The droplets of several o/w emulsions also contain fat crystals, at least at low temperatures, and are thus—strictly speaking—not emulsions.

2. *Droplet size distribution*. This has an important bearing on physical stability, smaller drops generally giving more stable emulsions. However, the energy and the amount of emulsifier needed to produce an emulsion increase with decreasing droplet size. A typical mean droplet diameter is 1 μm, but it can range between 0.2 and several μm. Because of the great dependence of stability on droplet size, the width of the size distribution is also important. Examples of droplet size distributions are in Figure 13.25a.
3. *Volume fraction* of dispersed phase (ϕ). In most foods, ϕ is between 0.01 and 0.4. For mayonnaise it may be 0.8, that is, above the value for maximum packing of rigid spheres, roughly 0.7; this means that the oil droplets are somewhat distorted. The volume fraction has a large effect on emulsion viscosity, ranging from a thin liquid to a kind of paste, as ϕ increases.
4. Composition and thickness of the *surface layer* around the droplets. This determines interfacial properties and colloidal interaction forces (Section 13.2.7); the latter greatly affect physical stability.
5. Composition of the *continuous phase*. This determines solvent conditions for the surfactant, pH, and ionic strength, and thereby colloidal interactions. The viscosity of the continuous phase has a pronounced effect on creaming.

Unlike the solid particles in a suspension, emulsion droplets are spherical (greatly simplifying many predictive calculations) and deformable (allowing droplet disruption and coalescence). Moreover, their interface is fluid, allowing interfacial tension gradients to develop. Nevertheless, in most conditions, emulsion droplets behave like solid particles. From Equation 13.8, the Laplace pressure of a droplet of 1 μm radius and interfacial tension $\gamma = 5$ mN m^{-1} is 10^4 Pa. For a liquid viscosity of $\eta = 10^{-3}$ Pa s (water), and a velocity gradient achieved by agitation or flow ∇v of 10^5 s^{-1}

(this is very vigorous), the shear stress $\eta \cdot \nabla v$ acting on the droplet would be 10^2 Pa. This implies that the droplet deformation would be negligible. Moreover, the surfactant at the droplet surface allows this surface to withstand a shear stress (Section 13.2.6). For the conditions mentioned, an interfacial tension difference between two sides of the droplet of 1 mN m^{-1} would more than suffice to prevent lateral motion of the interface, and a difference of this magnitude can be achieved readily. It can be concluded that emulsion droplets behave like solid spheres, unless agitation is extremely vigorous or the droplets are very large.

13.6.2 Emulsion Formation [80,90,91]

In this section the size of the droplets and the surface load obtained during making of emulsions are discussed, especially when protein is used as the surfactant.

Droplet break-up. To make an emulsion, one needs oil, water, an emulsifier (i.e., a suitable surfactant), and energy (generally mechanical energy). Making drops is easy, but to break them up into small droplets generally is difficult. Drops resist deformation and thereby break-up because of their Laplace pressure, which becomes larger as droplet size decreases. This necessitates a large input of energy. The energy needed will be reduced if the interfacial tension, hence the Laplace pressure, is reduced by adding an emulsifier, though this is not the latter's main role.

The energy needed to deform and break up droplets is generally provided by intense agitation. Agitation can cause sufficiently strong viscous shear forces if the continuous phase is sufficiently viscous. This is the common situation when making w/o emulsions ($\eta_{oil} \approx 0.05$ Pa s), resulting in droplets with diameters down to a few μm. In o/w emulsions the viscosity of the continuous phase tends to be low and to break up droplets inertial forces are needed. These are produced by the rapid, intensive pressure fluctuations occurring in turbulent flow. The machine of choice is the high-pressure homogenizer, which can produce droplets as small as 0.1 μm. The average droplet size obtained is about proportional to the homogenization pressure to the power −0.6 (cf. Figure 13.25a). When using high-speed stirrers, faster stirring, longer stirring, or stirring in a smaller volume result in smaller droplets; however, average droplet diameters below 1 or 2 μm usually cannot be obtained.

There are, however, other factors that affect droplet size. Figure 13.26 depicts the *various processes* that occur during emulsification. Besides disruption of droplets (Figure 13.26a), emulsifier has to be transported to the newly created interface (Figure 13.26b). The emulsifier is not transported by diffusion, but by convection, and this occurs extremely fast. The intense turbulence (or the high shear rate, if that is the situation) also leads to frequent encounters of droplets (Figure 13.26c and 13.26d). If these are as yet insufficiently covered by surfactant, they may recoalesce (Figure 13.26c). All these processes have their own time scales, which depend on several conditions, but a few μs is fairly characteristic. This means that all processes occur numerous times, even during one passage through a homogenizer valve, and that a steady state—where break-up and coalescence balance each other—is more or less attained.

The main role of the emulsifier is to *prevent recoalescence* of newly formed droplets. It may seem logical to attribute this function to colloidal repulsion between the droplets, caused by the adsorbed surfactant. However, the droplets are repeatedly pressed together due to the agitation, whether in turbulent or in laminar flow. The maximum stress involved is of the order of the stress needed to break up the droplets, which is of the order of their Laplace pressure, which would be, say, 10^4 Pa. Sample calculations show that the "disjoining" pressure between droplets due to colloidal repulsion will generally be far smaller, say, 10^2 Pa or less. Hence, this would be insufficient to prevent the drops coming close and would therefore not prevent their recoalescence. Indeed, experimental work often shows poor correlation between recoalescence during emulsification and coalescence in the finished emulsion.

The mechanism involved in preventing recoalescence is probably as follows. When two droplets that are (partially) covered by surfactant are pressed together, the liquid between them will be

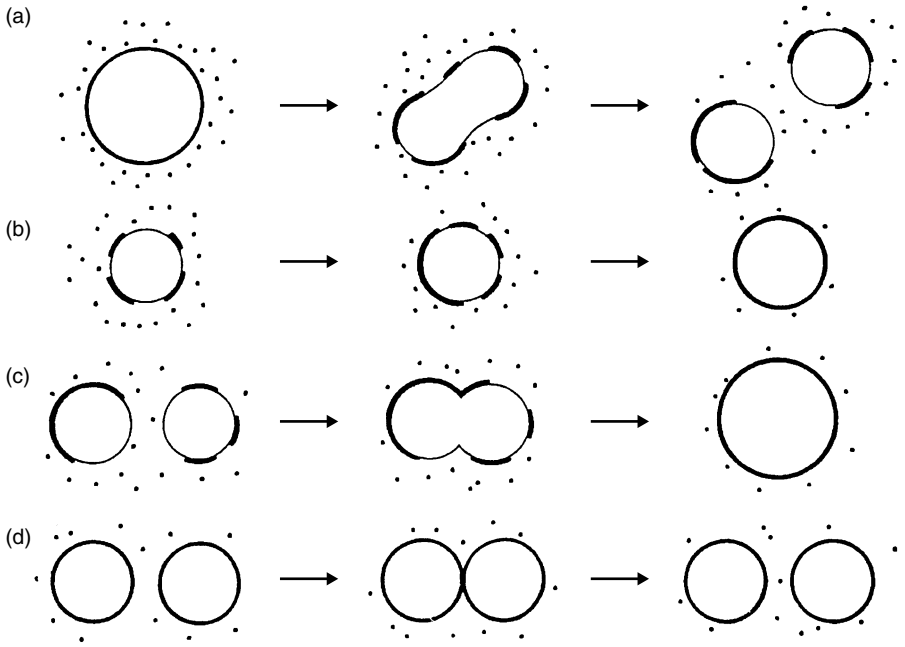

FIGURE 13.26 Important processes occurring during emulsification. The drops are indicated by thin lines and the emulsifier by heavy lines and dots. Highly schematic and not to scale. (Walstra, P. (2003), *Physical Chemistry of Foods*, Marcel Dekker, New York.)

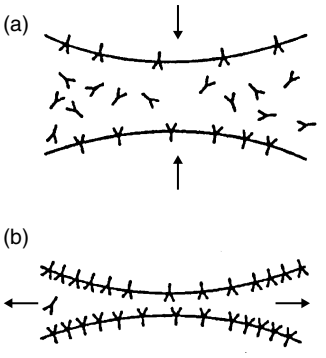

FIGURE 13.27 Two drops approach each other during emulsification. Illustration of the formation of a γ-gradient that slows down the outflow of continuous phase from the gap between the droplets. Surfactant molecules indicated by Y. (Modified after Walstra, P. (1993), *Chem. Eng. Sci.* **48**: 333–349.)

squeezed out, causing the formation of an interfacial tension gradient. This is illustrated in Figure 13.27. The γ-gradient will then cause considerable slowing down of the liquid flow as discussed in relation to Figure 13.9a and 13.9d. This can strongly decrease the rate of approach of the droplets. It would not prevent them to come very close, but the stress pushing them together will generally be short-lived or even change sign (i.e., pulling the drops apart) before the droplets can coalesce. Sample calculations confirm that the stresses and the time scales involved are of the right order of magnitude.

The phenomenon is often called the *Gibbs–Marangoni effect*: its magnitude depends on the value of the Gibbs elasticity of the film (i.e., twice the local surface-dilational modulus) and the mechanism is related to the Marangoni effect.

Bancroft's rule. This rule states: when making an emulsion of oil, water, and a surfactant (emulsifier), the continuous phase will become the one in which the surfactant is best soluble. In Figure 13.27, the surfactant is present in the continuous phase. If it would be in the droplets, a γ-gradient can hardly develop, since surfactant molecules can readily reach the interface, which will result in an adsorption layer of almost constant composition. If the surfactant is in the continuous phase, the thin film between the approaching droplets will soon be (almost) depleted of surfactant, and a γ-gradient can persist. Hence, when the formation of w/o emulsion is desired, a surfactant with a small HLB number is needed; for an o/w emulsion, one with a high HLB number.

Proteins are the emulsifiers of choice for o/w food emulsions because they are eatable, surface active, water soluble, and provide superior resistance to coalescence [86]. However, at equal agitation intensity, the droplets obtained are substantially larger than for a suitable small-molecule surfactant at the same mass concentration. The main reason can be derived from Figure 13.3b. It is seen that for a protein, a much higher surface excess concentration (Γ) at the o/w interface is needed to obtain a significant lowering of γ than for SDS. This also means that the possibility to form a significant γ-gradient during emulsification is far smaller than for SDS (and most other small-molecule emulsifiers). This implies, in turn, that the extent of droplet recoalescence will be much stronger than for SDS, as is indeed experimentally observed.

However, droplets can be made smaller by applying more intense emulsification, for example, a higher homogenization pressure, provided that sufficient protein is present. Some examples on average droplet size (d_{vs}) are given in Figure 13.25b. At a high emulsifier concentration, d_{vs} reaches a plateau value. This value is smaller for the nonionic surfactant than for the proteins, for the most part because the former produces a lower interfacial tension.

It is seen that the various proteins give about the same plateau value for d_{vs}. This is not strange because they produce comparable values for the interfacial tension (about 10 mN m^{-1}). But at low concentrations large differences in d_{vs} are apparent. Several tests have been developed to evaluate the suitability of proteins as emulsifiers. The well-known emulsifying activity index (EAI) involves emulsifying a large quantity of oil in a dilute protein solution [55]. This test corresponds roughly to the conditions indicated by the broken line in Figure 13.25b. Hence, the test result is not realistic for most practical situations, if only because the protein/oil ratio normally would be much larger. Consequently, EAI values are often irrelevant.

Attempts have been made to explain differences in EAI of various proteins by differences in their surface hydrophobicity [33]. However, the correlation is poor and other workers have refuted this concept (e.g., [59]). In the authors' view, proteins differ primarily in emulsifying efficiency because they differ in molar mass or in solubility. For a larger molar mass and the same mass concentration, the molar concentration is smaller, and the latter is presumably the most important variable in providing a strong Gibbs–Marangoni effect. Proteins of a smaller molar mass would thus be more efficient emulsifiers. It should be realized that several protein preparations, especially industrial products, contain molecular aggregates of various size, thereby greatly increasing effective molar mass and decreasing emulsifying efficiency. By and large, protein preparations that are poorly soluble are poor emulsifiers.

A substantially decreased molar mass, as realized, for instance, by partial hydrolysis of the protein, will indeed lead to the formation of smaller droplets. On the other hand, emulsions obtained with fairly small peptides usually show significant coalescence [61].

Another important variable is the *surface load* (Γ). If an emulsifier tends to give a high Γ, relatively much of it is needed to produce an emulsion. Compare, for instance, whey protein and soya protein in Figures 13.25b and 13.28. Moreover, a fairly high Γ is usually needed to obtain a stable emulsion.

In the case of small-molecule surfactants, equilibrium is reached between Γ and bulk concentration of surfactant. Consequently, knowledge of total surfactant concentration, o/w interfacial area, and of the adsorption isotherm (e.g., Figure 13.3a) will allow calculation of Γ, irrespective of the manner of formation of the emulsion. This is not the case when a protein (or other polymer) is

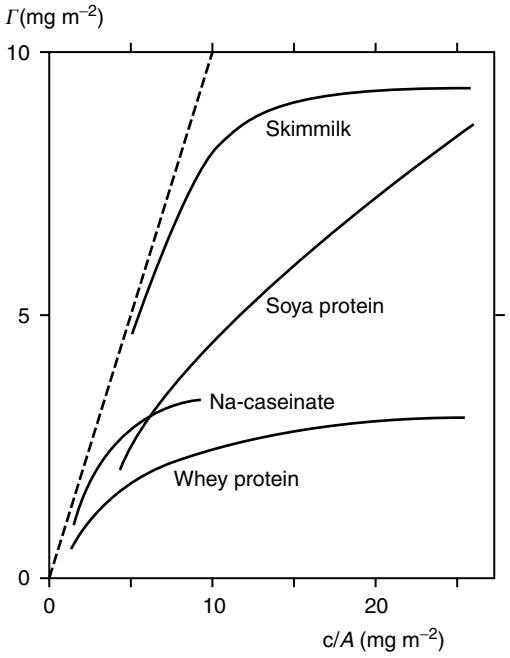

FIGURE 13.28 Protein load (Γ) as a function of the protein concentration (c) per unit oil surface area (A) created by emulsification. The broken line indicates the relation that would be obtained if all of the protein present would adsorb. (Walstra, P. and A.L. de Roos (1993), *Food Rev. Intern.* **9**: 503–525.)

the emulsifier, because thermodynamic equilibrium is not reached (Section 13.2.2). Thus, the surface load of a protein can depend on the way of making the emulsion, in addition to the variables mentioned.

It has been observed that plots as given in Figure 13.28 are more suitable to relate Γ to protein concentration. The figure gives some examples of results obtained for various proteins. If c/A is very small, some proteins presumably almost fully unfold at the o/w interface and form a stretched polypeptide layer, with a Γ of about 1 mg m^{-2}. Several highly soluble proteins give plateau values of about 3 mg m^{-2}. Aggregated proteins can yield much larger values. It may also be noted that any large protein aggregates present tend to become preferentially adsorbed during emulsification, thereby further increasing Γ.

An emulsifier is needed not only for the formation of an emulsion, but also for *providing stability* of the emulsion once made. It is of some importance to clearly distinguish between these main functions, since they often are not related. An emulsifier may be very suitable for making small droplets, but may not provide long-term stability against coalescence, or vice versa. Evaluation of proteins merely for their ability to produce small droplets is, therefore, not very useful. Another, often desirable, feature of a surfactant is to prevent aggregation under a range of conditions (pH near the isoelectric point, high ionic strength, poor solvent quality, high temperature). Types of emulsion instability and means of prevention are discussed next.

13.6.3 Types of Instability [19,51,85,86]

Emulsions can undergo several types of physical change as illustrated in Figure 13.29. The figure pertains to o/w emulsions, the difference with w/o emulsions is that downward sedimentation rather than creaming would occur.

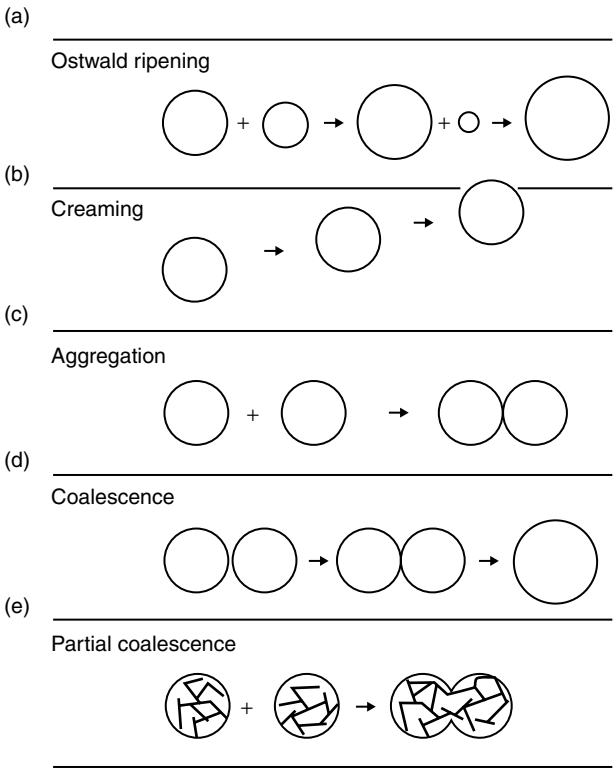

FIGURE 13.29 Types of physical instability for oil-in-water emulsions. Highly schematic. The size of the contact area in (d) may be greatly exaggerated; the short heavy lines in (e) denote triacylglycerol crystals.

Ostwald ripening (Figure 13.29a) does not normally occur in o/w emulsions, because triacylglycerol oils are commonly used and they are insoluble in water. When essential oils are present (e.g., in citrus juices), some have sufficient solubility so that smaller droplets gradually disappear [23]. W/O emulsions may exhibit Ostwald ripening. Data in Table 13.3 show only a very small solubility excess for a 2 μm droplet, but it would be sufficient to produce marked Ostwald ripening during prolonged storage. This can easily be prevented by adding a suitable solute to the water phase (i.e., one that is insoluble in oil). A low concentration of salt (say, NaCl) will do: as soon as a small droplet shrinks, its salt concentration and osmotic pressure increase, thereby producing a driving force for water transport in the opposite direction. The net result is a stable droplet size distribution.

The other instabilities are discussed in other sections: creaming in 13.4.2, aggregation in 13.4.3, coalescence in 13.6.4, and partial coalescence in 13.6.5.

The various changes may affect each other. Aggregation greatly enhances creaming and if this occurs, creaming further enhances aggregation rate, and so on. Coalescence can only occur when the droplets are close to each other (i.e., in an aggregate or in a cream layer). If the cream layer is more compact, which may occur when fairly large separate droplets cream, coalescence will be faster. If partial coalescence occurs in a cream layer, the layer may assume characteristics of a solid plug.

It is often desirable to *establish the kind of instability* that has occurred in an emulsion. Coalescence leads to large drops, not to irregular aggregates or clumps. Clumps due to partial coalescence will coalesce into large droplets when heated sufficiently to melt the fat crystals. A light microscope can be used to establish whether aggregation, coalescence, or partial coalescence has occurred. Section 13.4.3 gives some hints for distinguishing among various causes of aggregation. It is fairly common that coalescence or partial coalescence leads to broad size distributions, and then the larger droplets or clumps cream very rapidly.

Agitation can disturb creaming and may disrupt aggregates of weakly held droplets, but not clumps formed by partial coalescence. Slow agitation tends to counteract true coalescence.

If air is beaten in an o/w emulsion, this may lead to adsorption of droplets onto air bubbles. The droplets may then be disrupted into smaller ones, due to spreading of oil over the a/w interface (Section 13.2.3). If the droplets contain crystalline fat, clumping may occur; beating in of air thus promotes partial coalescence. This is what happens during churning of cream to make butter and also during whipping of cream. In the latter case, the clumped, partially solid droplets form a continuous network that encapsulates and stabilizes the air bubbles, and lends stiffness to the foam.

A way to prevent or retard all changes except Ostwald ripening is to *immobilize the drops*, for instance, by causing the continuous phase to gel (Section 13.5.2). Examples are butter and margarine. Here, the water droplets are immobilized by a network of fat crystals. Moreover, some crystals become oriented at the oil–water interface because of the favorable contact angle (Section 13.2.3). In this way, the droplets cannot closely encounter each other. If the product is heated to melt the crystals, the aqueous droplets readily coalesce. Often, a suitable surfactant is added to margarine to prevent rapid coalescence during heating, since this would cause undesirable spattering.

13.6.4 Coalescence [25,85,86]

This discussion will focus on o/w emulsions. The theory is still in a state of some confusion.

Film rupture. Coalescence is induced by rupture of the thin film (lamella) between close droplets (the same applies for a film between gas bubbles). This is illustrated in Figure 13.30a: a small hole can somehow be formed in the film by chance (due to Brownian motion). If the radius of the hole is larger than half the film thickness ($R > \delta/2$), the Laplace pressure near 1 is larger than near 2; hence, liquid in the film will flow from 1 to 2; hence, the hole will expand, which implies that the film ruptures; hence, the droplets will immediately flow together. It also would follow that any film will rupture if it becomes thin enough; colloidal repulsion will oppose this. However, Figure 13.30b illustrates that the film may become locally thinner because symmetric waves can develop at the film surfaces. The amplitude of the waves can be larger, hence film rupture more likely, when the wavelength of the film is greater (i.e., the film area is larger) and γ is smaller.

We will not further discuss the underlying theory, also because the theory does not explain all observations on droplet coalescence. For instance, it is observed that some macromolecular surfactants cause an additional resistance to coalescence. Presumably, these molecules form coherent monolayers that also have to rupture for the film to rupture. Important examples are proteins that tend

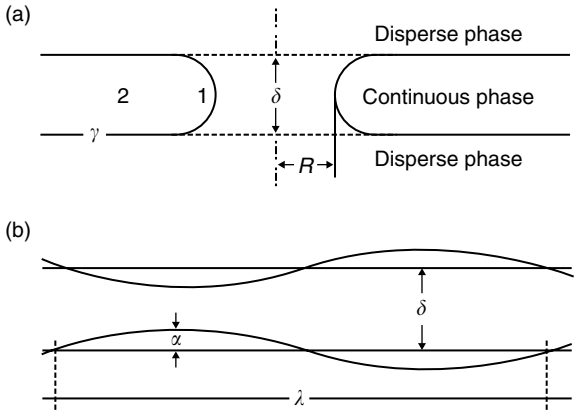

FIGURE 13.30 Cross section of part of a film of (average) thickness δ between drops (or gas bubbles). (a) Illustration of hole formation. (b) Properties of a symmetric wave developing on the film.

to form coherent layers after adsorption, such as β-lactoglobulin; here, formation of intermolecular −S−S− bonds appears to be involved.

Film rupture is thus a chance event and this has important consequences: (1) The probability of coalescence, if it does occur, will be proportional to the time that the droplets are close to each other. Hence, it is especially likely in a cream layer or in aggregates. (2) Coalescence is a first-order rate process with respect to time, unlike aggregation, which is in principle second order. (3) The probability that rupture of a film occurs will be proportional to its area. This implies that flattening of the droplets on approach, leading to the formation of a greater film area, will promote coalescence.

Whether or not a flat film formed is, therefore, an essential variable. It can be expressed in a *Weber number*, which gives the ratio of the local stress on a droplet pair over the Laplace pressure of the drop. The local stress is the external stress (σ_{ext}) times a stress concentration factor; the latter equals droplet radius over the smallest distance (h) between the drop surfaces. This leads to

$$\mathrm{We} = \frac{\sigma_{ext} d^2}{8\gamma h} \tag{13.30}$$

If We > 1, a flat film is formed between the drops, and the larger We, the larger the film radius. For We $\ll 1$, there is no real film formed and there is virtually no coalescence.

The external stress can be due to colloidal attraction forces; or to hydrodynamic stresses caused by flow or agitation; or to the gravitational or centrifugal stress in the cream layer (or sediment). For small protein-covered emulsion droplets, the condition We $\ll 1$ is nearly always fulfilled, unless strong external forces are acting. Even in a cream layer formed under gravity, the external stress is small enough for We to be much below unity.

Taking the above considerations into account, we can draw the following conclusions. Coalescence is less likely for

1. *Smaller droplets*. (1) They lead to a smaller We, hence to a smaller film area between droplets, hence to a lower probability of rupture of the film. (2) More coalescence events are needed to obtain droplets of a certain size. (3) The rate of creaming is decreased. In practice, average droplet size is often the overriding variable.
2. *A thicker film between droplets*. This implies that strong or far reaching repulsive forces between droplets (Section 13.3) provide stability against coalescence. For DLVO-type interactions, coalescence will readily occur if the droplets are aggregated in the primary minimum (Figure 13.11). Steric repulsion is often especially effective against coalescence, because it tends to keep the droplets relatively far apart.
3. *A greater interfacial tension*. This may appear strange, because a surfactant is needed to make an emulsion and a surfactant decreases γ. Moreover, a smaller γ implies a smaller surface free energy of the system; hence, a smaller driving force for coalescence. However, it is the *activation* free energy for film rupture that counts, which is larger for a larger γ. This is because a larger γ makes it more difficult to form and to deform a film (bulging, development of a wave on it), and local deformation is needed to induce rupture.

Based on these principles, *proteins* appear to be very suitable for preventing coalescence, and this agrees with observation. Proteins do not produce a very small γ, and they often provide considerable repulsion, both electric and steric. Figure 13.31 shows results of experiments in which small droplets in an extremely dilute protein solution were allowed to cream to planar o/w interfaces of a given age, and the time needed for coalescence was observed. A strong effect of droplet size is apparent. The results shown were obtained under conditions (protein concentration and adsorption time) where the surface load of the proteins would have been at most about 0.5 mg m^{-2}, implying very weak repulsion. In cases where a thicker adsorbed layer was allowed to form, the authors observed virtually no coalescence.

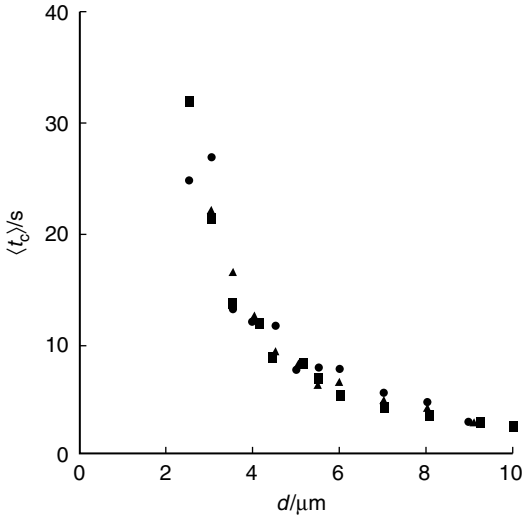

FIGURE 13.31 Average time ($\langle t_c \rangle$) for coalescence of oil droplets of various diameter (d) with a plane o/w interface, in 20-min-old 1 ppm protein solutions. ▲, β-casein; ●, κ-casein; ■, lysozyme. (After Dickinson, E., B.S. Murray and G. Stainsby (1988), *J. Chem. Soc. Faraday Trans. 1* **84**: 871–883.)

Figure 13.31 shows no significant difference between proteins in their ability to prevent coalescence. This is also the general experience in practice, except for gelatin, which is somewhat less effective than most other proteins. Under severe conditions (see below), differences among proteins may be observed, with caseinates tending to be superior. Partial hydrolysis of proteins can significantly impair their ability to prevent coalescence [61].

Attempts have been made to relate the coalescence inhibiting ability of proteins (and of other surfactants) to various properties, particularly surface shear viscosity (Section 13.2.5) of the adsorbed protein layer. In some cases, a positive correlation between (apparent) surface shear viscosity and coalescence stability is observed, but there are several cases where deviation from this relationship are very large; for example, caseinates give a very low surface shear viscosity, but quite good coalescence stability. Reasonable correlations are observed for many globular proteins if the drops are relatively large or Γ is fairly small; the cause for the increase in stability is presumably that intermolecular cross-links are formed at the interface, as mentioned above.

On the other hand, in a highly concentrated emulsion (e.g., $\phi = 0.8$) that is subjected to strong elongational flow by pressing the emulsion through a small orifice, considerable coalescence can occur if the surface layers consist of cross-linked globular proteins [70]. It has been explained by considering the emulsion as a soft solid that is subject to macroscopic fracture (cf. Section 13.5.1). Apparently, the fracture planes are through the emulsion droplets, leading to strong local coalescence. High-ϕ emulsions stabilized with caseinate, which does not form strong intermolecular cross-links, do not show significant coalescence in elongational flow.

Most *small-molecule surfactants* yield a small interfacial tension. Because a small γ favors coalescence, surfactants that provide considerable steric repulsion, such as the Tweens, are among the most effective. Ionic surfactants are effective against coalescence only at low ionic strength.

Small-molecule surfactants present in (or added to) protein-stabilized emulsions tend to displace protein from the droplet surface (Section 13.2.2; Figure 13.5), and this generally decreases resistance to coalescence. If coalescence is desired, this provides a method to achieve it; for example, add SDS and some salt (to decrease double layer thickness), and rapid coalescence will usually occur.

Food emulsions may exhibit coalescence under *extreme conditions*. For example, during freezing, formation of ice crystals will force the emulsion droplets closer together, often causing copious

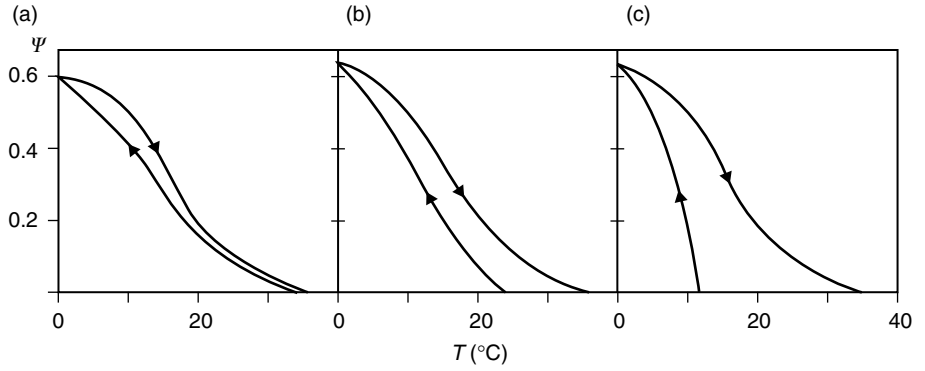

FIGURE 13.32 Proportion of milk fat being solid (ψ) after 24 h cold storage at temperature T, and after warming it again (after it had been kept at 0°C). (a) Fat in bulk, (b) same fat in natural cream (globule size about 4 μm), and (c) same fat in homogenized cream (globule size about 0.5 μm). (From results by P. Walstra and E.C.H. van Beresteyn (1975), *Neth. Milk. Dairy J.* **29**: 35.)

coalescence on thawing. Something similar happens upon drying and subsequent redispersion; here coalescence is alleviated by a relatively high concentration of "solids not fat." In such cases, best stability is obtained by having small droplets and a thick protein layer, for example, of Na-caseinate.

Another extreme condition is centrifugation. This causes a cream layer to form rapidly, and pressing the droplets together with sufficient force to cause a high We; hence, considerable flattening even of small droplets, and likely coalescence. This implies that centrifugation tests to predict coalescence stability of emulsions during storage are usually not valid, since the conditions during centrifugation differ so greatly from those during handling of emulsions. (This does not mean that centrifugation tests to predict creaming are useless. They can be quite helpful if the complications discussed in Section 13.4.2 are taken into account.)

Predicting coalescence rate is always very difficult. The best approach is to use a sensitive method to estimate average droplet size (e.g., turbidity at a suitable wavelength) and establish the change over time (say, a few days).

13.6.5 Partial Coalescence [6–8,14,42,72,85]

In many o/w food emulsions part of the oil in the droplets can crystallize. The proportion of fat solid, ψ, depends on the composition of the triacylglycerol mixture and on temperature (Chapter 4). In emulsion droplets, ψ can also depend on temperature history, since a finely emulsified oil can show considerable and long-lasting undercooling, the more so for smaller droplets [81]. This is illustrated in Figure 13.32. If an emulsion droplet contains fat crystals, they usually form a continuous network. These phenomena greatly affect emulsion stability. The presence of crystals means that we have no true o/w emulsion and it may be better to speak of fat globules.

Fat globules containing a network of fat crystals cannot fully coalesce (Figure 13.27e). If the film between globules ruptures, they form an irregular clump, held together by a "neck" of liquid oil. True and partial coalescence thus have different consequences. Partial coalescence causes an increase in the apparent volume fraction of dispersed material, and if the original volume fraction is about 0.2 or higher, and shear rate is fairly small, a solid, or gel-like network of partially coalesced clumps can form.

Rupture of the film between close globules can be triggered by a crystal that protrudes from the globule surface and pierces the film. This happens particularly during flow or agitation, and then it may occur, say, six orders of magnitude faster than true coalescence (same emulsion, no fat crystals). This implies that partial coalescence is far more important than true coalescence, if an o/w emulsion is subject to fat crystallization.

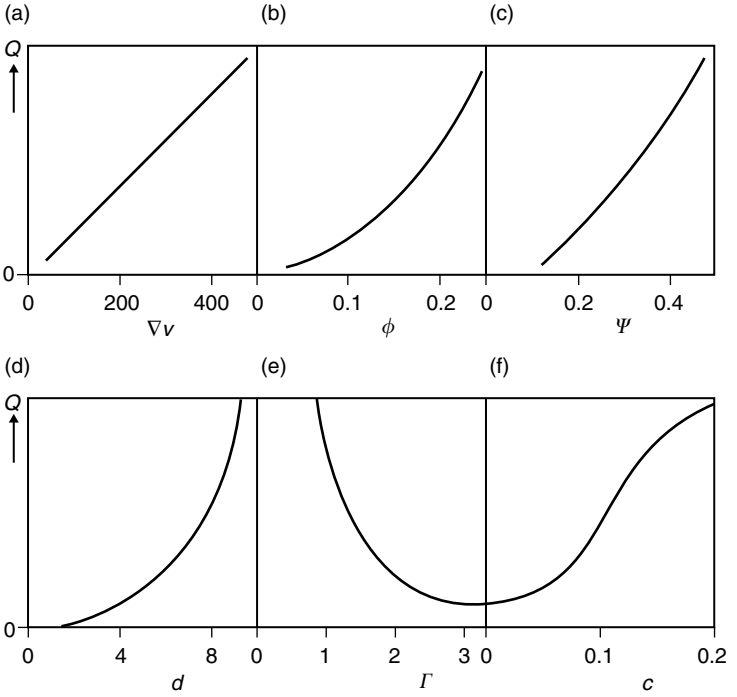

FIGURE 13.33 Approximate results obtained on the rate of partial coalescence (Q) in protein-stabilized emulsions as a function of (a) shear rate ∇v (s^{-1}); (b) volume fraction ϕ; (c) proportion of fat solid ψ; (d) average globule diameter d (μm); (e) protein surface load Γ (mg m^{-2}); and (f) concentration of added small-molecule surfactant c (%). Only meant to illustrate trends. (Walstra, P. (1996), in *Encyclopedia of Emulsion Technology, Vol. 4* (P. Becher, ed.), Dekker, New York, pp. 1–62.)

The kinetics of partial coalescence is complicated and variable, because it is affected by several factors. In many emulsions, large particles (original globules or clumps already formed) are more prone to partial coalescence than small ones, leading to a self-accelerating process, which soon leads to large clumps that cream rapidly. The remaining layer may then exhibit a decreased average particle size. Other emulsions may simply show a gradual increase in average particle size with time.

The most important factors affecting the *rate of partial coalescence* generally are as follows (Figure 13.33):

1. *Shear rate*. This has various effects: (1) The encounter rate between particles is proportional to shear rate (Section 13.4.3). (2) Because of the shear flow, two globules that encounter each other will roll over each other, thereby significantly enhancing the probability that a crystal protruding from one globule attains—for a short while—a position where it is close to the other globule. (3) The shear force tends to press approaching globules closer together against any repulsive force acting between the globules, thereby enhancing the possibility that a protruding crystal at a favorable position can pierce the film. Thus, flow rate has a very large effect on the rate of partial coalescence, and this influence is even stronger when the flow is turbulent rather than laminar.
2. *Volume fraction of droplets*. For a higher ϕ, the rate of partial coalescence is obviously greater, being about second order with respect to ϕ.
3. *Fat crystallization*. If the fraction solid, ψ, is zero, partial coalescence is impossible and it can also not occur in the absence of liquid fat ($\psi = 1$). For fairly low ψ, partial coalescence rate generally increases with increasing ψ, as this causes more crystals to

protrude. However, the relation between ψ and rate is variable, largely due to variation in crystal size and arrangement. An important aspect is that the crystals must form a network throughout the globule to support protruding crystals. The minimum ψ needed for such a network often is of the order 0.1. If most of the oil is crystallized and the crystals are very small, the crystal network may tenaciously hold the remaining oil, thereby preventing partial coalescence, even if the film is pierced. Moreover, the protrusion distance may depend on ψ, temperature history, crystal size, and crystal shape.

4. *Globule diameter*. A relation as depicted in Figure 13.33d is usually observed, but the scale of globule size varies substantially among emulsions. The effect of d presumably is due to (1) larger globules sensing a larger shear force and (2) larger globules exhibiting a larger film area between two globules.
5. *Surfactant type and concentration*. Two effects are of major importance. First, these variables will determine the oil–crystal–water contact angle (Section 13.2.3) and thereby affect the distance a given crystal can protrude. Second, these variables determine repulsion (strength and range) between the globules. The weaker the repulsion, the easier it is for two droplets to closely approach each other, thereby increasing the likelihood that a protruding crystal will pierce the film between them. The repulsion, together with the globule size, will thus determine what minimum shear rate is needed for partial coalescence to occur; values between 5 and 120 s^{-1} have been observed. Some emulsions show no partial coalescence at all at the shear rates studied. The best type of surfactant to achieve this is, again, a protein, if the surface load is high enough (Figure 13.33e). Addition of a small-molecule surfactant generally leads to displacement of the protein from the surface (Section 13.2.2), thereby greatly enhancing partial coalescence (Figure 13.33f).

Ice cream [51,89]. We will illustrate some consequences of partial coalescence in relation to making and properties of ice cream. This product has an intricate structure. It contains ice crystals, an aqueous phase that consists of concentrated skim milk with added sugar(s), air bubbles, and milk fat globules, which are partially crystalline. The air bubbles and the ice crystals are made in a scraped-surface heat exchanger, during vigorous agitation and rapid cooling. The fat globules are needed for covering the air bubbles to ensure stability against Ostwald ripening and coalescence of the bubbles (Section 13.7.2).

The fat globules show extensive partial coalescence in the heat exchanger, which is desirable. It leads to the formation of a space-filling network of fat globule clumps and fat globule-covered air bubbles. This structure provides a "melt-down resistance," that is, a certain firmness remains after the ice crystals have melted in the mouth. Moreover, it gives the product a "dry" appearance and a "short" consistency; the latter greatly diminishes the stickiness of the product. These properties make the ice cream more attractive for the consumer and allow the use of fast packaging machines.

If ice cream is made of natural (unhomogenized) cream (plus sugar and a number of additives), partial coalescence happens extremely fast. The natural milk fat globules are not very small (for the most part 1.5–6 μm) and have a surface layer that gives a quite low interfacial tension (1–1.5 mN m^{-1}). Moreover, the vigorous agitation causes large clumps of fat globules to form, far too large to be able to fully cover the air bubbles desired. Consequently, the ice cream obtained has a coarse structure, with large air bubbles and large fat clumps. The remedy is to homogenize the cream, which results in far smaller fat globules (say, 0.4–1.2 μm). The surface layers of these globules largely consist of milk plasma proteins. This inhibits partial coalescence (Figure 13.33d and 13.33e). The product obtained has small air bubbles and a homogeneous structure, but not the desired melt-down resistance and dryness.

To overcome the latter problem, small-molecule surfactants are added that substantially enhance partial coalescence rate (Figure 13.33f). By varying type and concentration of these surfactants, optimum conditions for obtaining a product of good quality can be established.

TABLE 13.7
Comparison of Foams and Emulsions: Order of Magnitude of Some Quantities

Property	Foam	Foam	Emulsion w/o	Emulsion o/w	Units
Drop/bubble diameter	10^{-3}	10^{-4}	5×10^{-6}	10^{-6}	m
Volume fraction	0.9	0.8	0.1	0.1	–
Drop/bubble number	10^{9}	10^{11}	10^{15}	10^{17}	m^{-3}
Interfacial tension	0.05	0.05	0.005	0.01	$N\ m^{-1}$
Laplace pressure	2×10^{2}	2×10^{3}	4×10^{3}	4×10^{4}	Pa
Solubility D in C	2.1[a]	2.1[a]	0.15	0	vol.%
Density difference D–C	-10^{3}	-10^{3}	10^{2}	-10^{2}	$kg\ m^{-3}$
Viscosity ratio D/C	10^{-4}	10^{-4}	10^{-2}	10^{2}	–
Time scale[b]	10^{-3}	10^{-4}	10^{-5}	10^{-6}	s

Key: D, disperse phase (air, triacylglycerols, or water); C, continuous phase.
[a] If it concerns CO_2, the solubility is about 100 vol.% at a pressure of 1 bar.
[b] Characteristic times during formation.

13.7 FOAMS

In a sense, foams are much like o/w emulsions; both are dispersions of a "hydrophobic" fluid in a hydrophilic liquid. However, because of considerable quantitative differences, their properties are also qualitatively different. Quantitative information is given in Table 13.7. It is evident that bubble diameter is so large as to exclude foam bubbles from the realm of colloids. Large diameter combined with large density difference, causes foam bubbles to cream faster than emulsion droplets by some orders of magnitude. The relatively high solubility of air in water can cause rapid Ostwald ripening (often called disproportionation in foams). If the gas phase is CO_2, as it is in some foods (bread, carbonated beverages), the solubility is even higher, by a factor of about 50. The characteristic time scales during formation are two or three orders of magnitude longer for foams than for most o/w emulsions. Because creaming and Ostwald ripening occur so fast, physical instabilities often occur already during foam formation, which complicates the study of foaming and foam stability.

Several aspects of foams will be briefly discussed. Surface phenomena are of overriding importance to foam formation and properties; background information is provided in Section 13.2. Further sources of literature are [28,56,82,90]. For churning and whipping of cream, see [51,69]. Also some of the books mentioned in the bibliography, especially Walstra's and Dickinson's, have chapters on foam.

13.7.1 Formation and Description

In principle, foams can be made in two ways, by supersaturation or mechanically.

13.7.1.1 Via Supersaturation

A gas, usually CO_2 or N_2O, because of their high solubility, is dissolved in an aqueous liquid at high pressure (a few bars). When the pressure is released, gas bubbles form. These do not form by nucleation; a spontaneously formed gas bubble would need to have an initial radius of about 2 nm, which would imply a Laplace pressure (Equation 13.8) of about 10^{8} Pa, or 10^{3} bar. To achieve this, the gas would have to be brought to this pressure, which is, of course, impractical. Instead, gas bubbles always grow from small air pockets that are already present at the wall of the vessel or on small particles. The contact angle gas/water/solid may be as high as 150° for a fairly hydrophobic

solid, and this allows small air pockets to remain in crevices or sharp dents in the solid (Figure 13.7b). For a negative curvature, air can even remain there if less than saturated.

To give an example, if a pressurized vessel of a carbonated liquid is opened, the overpressure is released, CO_2 becomes supersaturated, and it diffuses toward any air pockets present. These grow and become dislodged when large enough, leaving a remnant from which another bubble can grow. The bubbles rise while growing further, and a creamed layer of bubbles is formed (i.e., a foam). These bubbles are always fairly large, say, 1 mm.

Another example is the formation of CO_2 in a leavened dough. Excess CO_2 collects at sites of entrapped small air bubbles, and these sites grow in size. Some of them grow to form visible gas cells, creating a macroscopic foam structure.

13.7.1.2 By Mechanical Forces

A gas stream can be led through narrow openings into the aqueous phase (sparging); this causes bubbles to form, but they are fairly large, generally >1 mm. Smaller bubbles can be made by beating air into the liquid. At first, large bubbles form and these are broken up into progressively smaller ones. Shear forces are typically too weak to obtain small bubbles and the break-up mechanism generally involves pressure fluctuations in a turbulent field, as is the case during the formation of o/w emulsions (Section 13.6.2). Bubbles of about 100 μm can be obtained in this way, the smallest ones being, say, 20 μm.

Beating is the method of choice in industrial processing. If this occurs in an open system, as when whipping egg white in a bowl, the main resultants of the process are the average bubble size and the volume fraction of gas incorporated ϕ. The latter is often expressed as *percent overrun*, which is equal to $100\phi/(1-\phi)$. The factors determining the overrun are insufficiently understood. Therefore, we will not discuss all aspects but give some important variables in Figure 13.34. The same variables affect the resulting bubble size; by and large, a higher beating speed and a higher surfactant concentration result in smaller bubbles. In industrial practice, closed systems are often used, implying that the amounts of liquid and gas can be metered. This then determines the overrun obtained, provided that enough surfactant is present.

To make a foam, a *surfactant* is needed. Almost any type will do, since the only criterion for its functionality is that a certain γ-gradient be created. This does not mean that any surfactant is

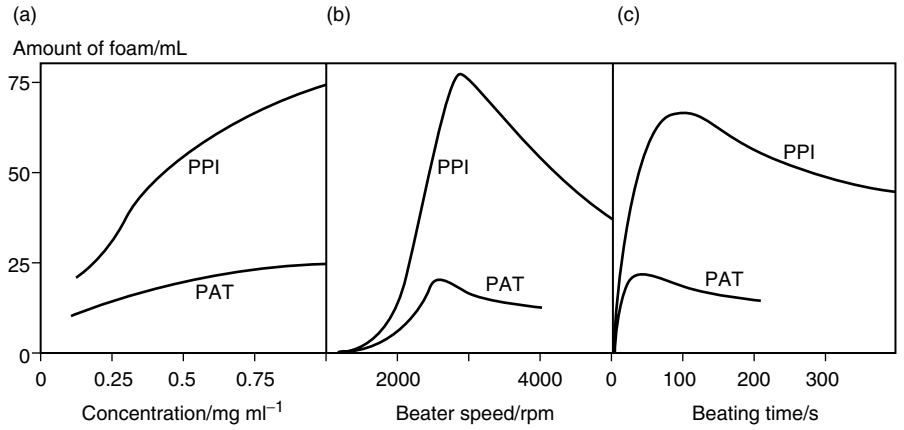

FIGURE 13.34 Amount of foam produced from dilute solutions of potato protein isolate and purified patatin. pH = 7.0, ionic strength = 0.05 molar. (a) Effect of protein concentration. (b) Effect of beater speed (revolutions per minute). (c) Effect of beating time. Results by courtesy of G. van Koningsveld. (Walstra, P. (2003), *Physical Chemistry of Foods*, Marcel Dekker, New York.)

suitable to make a stable foam, as will be discussed later. Moreover, it is the molar concentration of surfactant that determines the overrun, which means that proteins need higher mass concentrations than small-molecule amphiphiles.

Nevertheless, proteins are the agents of choice in the food industry: they are eatable and tend to give relatively stable foams. As seen in Figure 13.34a, the protein concentration is an important variable. To obtain a high overrun, much higher concentrations are needed. One of the reasons why egg white is a superior foaming agent, is that it contains 10% protein. A 5% solution of undenatured whey protein can yield an overrun of, say, 1000%. However, considerable variation exists among proteins in the concentration needed to obtain a given overrun. Some peptides obtained by hydrolysis of a protein can give a higher overrun than the protein itself at the same mass concentration, but the physical stability of the foam often is significantly impaired. As a rule of thumb, mixtures of proteins, or of proteins and peptides, are superior to most pure proteins as foaming agents.

13.7.1.3 Foam Structure Evolution

Figure 13.35 illustrates the stages in foam formation after the creation of initial bubbles. As soon as beating stops, bubbles rise rapidly and form a foam layer (unless liquid viscosity is quite high). The buoyancy force soon is sufficient to cause mutual deformation of bubbles, causing the formation of flat lamellae between them. The stress due to buoyancy is roughly equal to $\rho_{water} gH$, where H is the height in the foam layer (i.e., about 100 Pa for $H = 1$ cm). However, there is marked stress concentration as spherical bubbles come into contact, and this means that bubbles with a Laplace pressure of 10^3 Pa would become significantly flattened. Further drainage of interstitial liquid causes the bubbles to attain a polyhedral shape. Where three lamellae meet (never >3, because that would be an unstable conformation), a prism-shaped water volume, bounded by cylindrical surfaces, is formed. This structural element is called a Plateau border. Generally, residual small bubbles soon disappear by Ostwald ripening. In this way, a fairly regular polyhedral foam is formed, somewhat resembling a honeycomb structure. In the lower part of a foam layer, bubbles remain more or less spherical.

As the foam keeps draining, its volume fraction of air increases, and a liquid layer forms beneath the foam. The Laplace pressure in the Plateau borders is lower than in the lamellae, and this causes liquid to flow to the Plateau borders. Because the latter are interconnected, they provide pathways through which the liquid can drain. As drainage continues, a ϕ value of 0.95 can readily be reached, which corresponds to an overrun of 1900%. Such a foam is not very substantial as a food. To avoid (excessive) drainage, small filler particles may be incorporated, but they should be hydrophilic;

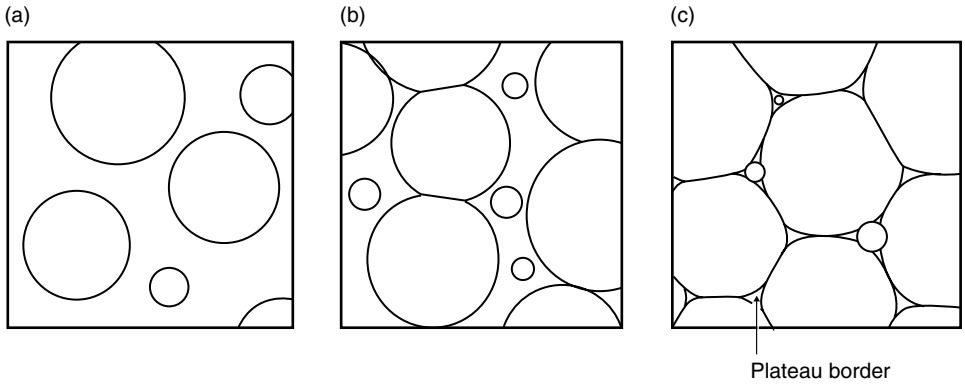

FIGURE 13.35 Subsequent stages (a, b, and c) in the formation of a polyhedral foam, once bubbles have been created. The thickness of the lamellae between bubbles are too small to be seen at this scale (bubble diameter <1 mm).

otherwise, considerable coalescence of bubbles can occur (Section 13.7.2.3). Small protein-coated emulsion droplets will function well, and they are incorporated in several whipped toppings. Another approach is gelation of the aqueous phase. This is employed in many aerated food products, such as meringues, foam omelets, bavarois, bread, and cakes. By letting the system gel in an early stage, it is also possible to make a foam with spherical bubbles; in other words a "bubbly" or "wet" foam, rather than a polyhedral or "dry" foam.

A polyhedral foam itself may be considered a gel. Deformation of the foam causes an increase in curvature of bubbles, a corresponding increase in Laplace pressure, and elastic behavior at small deformation. Then, at greater stress, bubbles slip past each other and viscoelastic deformation occurs. There is thus a yield stress (see Section 13.5.1), which is obvious, because even a tall portion of foam may retain its shape under its own weight. The yield stress usually exceeds 100 Pa.

13.7.2 Stability

Foams are subject to three main types of instability.

1. Ostwald ripening (disproportionation), which is the diffusion of gas from small to larger bubbles (or to the atmosphere). This occurs because the pressure in a small bubble is greater than in larger ones.
2. Drainage of liquid from and through the foam layer, due to gravity.
3. Coalescence of bubbles due to instability of the film between them.

These changes are to some extent interdependent: drainage may promote coalescence, and Ostwald ripening as well as coalescence may enhance drainage.

These instabilities are governed by fundamentally different factors, as will be made clear below. Unfortunately, many studies on foam stability have failed to distinguish between the three types of instability. One reason for this may be the lack of suitable methods to monitor bubble size distribution.

13.7.2.1 Ostwald Ripening

See Section 13.2 for fundamental aspects. Ostwald ripening is often the most important type of foam instability, especially in foods, where the bubble size is relatively small as compared to many other foams. Within minutes after foam formation, noticeable coarsening of the bubble size distribution often occurs. It happens most rapidly at the top of a foam layer, because the air can diffuse directly to the atmosphere and the layer of water between a bubble and the atmosphere is very thin, but also inside a foam Ostwald ripening can occur at a significant rate.

The classical treatment of Ostwald ripening rate, based on Equation 13.9 and the diffusion laws, is by de Vries [17]. He considered a small bubble of radius r_0 surrounded by much larger bubbles at an average distance δ. The change in radius with time t then would be given by

$$r^2(t) = r_0^2 - (RTDs_\infty\gamma/p\delta)t \tag{13.31}$$

where D is the diffusion coefficient of the gas in water (in $m^2\ s^{-1}$), s_∞ is solubility for $r = \infty$ (since the solubility of a gas is proportional to its pressure, it is given in mol $m^{-3}\ Pa^{-1}$), γ is interfacial tension (mostly about 0.05 N m^{-1}), and p is ambient pressure (often 10^5 Pa).

It follows from Equation 13.31 that a bubble will shrink ever faster as it becomes smaller. Furthermore, since γ and solubility for most gases in water are high, shrinkage is fast, as is illustrated by the following examples. A nitrogen bubble of radius 0.1 mm at $\delta = 1$ mm in water would disappear in about 3 min, and a similar CO_2 bubble in about 4 s. This is not quite realistic, since the geometric assumptions underlying Equation 13.31 are not fully met in practice, and also because the process is somewhat slower if a mixture of gasses, like air, is present. Moreover, as the smallest remaining

bubbles become larger, the rate of change decreases with time. Nevertheless, Ostwald ripening can occur quite fast.

Can Ostwald ripening be stopped or retarded? If a bubble shrinks, its area decreases and its surface load (Γ) increases, provided the surfactant does not desorb. If no desorption occurs, γ is lowered and thereby the Laplace pressure, which implies that the driving force for Ostwald ripening is decreased. It will even stop when the surface dilational modulus, E_{SD}, which is a measure of the change in γ with change in area (see Equation 13.10), becomes about equal to γ. However, surfactant normally desorbs and E_{SD} therefore decreases, at a rate that depends on several factors, especially surfactant type. For a foam made with small-molecule surfactants, desorption occurs readily, and retardation of Ostwald ripening tends to be negligible. Proteins, however, tend to desorb sluggishly (see Section 13.2.2), and E_{SD} may remain fairly high (Section 13.2.6), especially if the gas consists of CO_2. Ostwald ripening will then be substantially retarded [57], although shrinking bubbles (and emulsion droplets) may also collapse [22,45,51]. If the gas is air or N_2, implying that Ostwald ripening is much slower, E_{SD} tends to remain low, and Ostwald ripening is not substantially retarded.

Some proteins produce tenacious layers at the a/w interface because of cross-linking reactions between adsorbed molecules. Egg white especially is a good foam stabilizer. During beating, strong surface denaturation occurs, leading to fairly large protein aggregates. These remain irreversibly adsorbed, resulting in strong resistance to Ostwald ripening. Something similar can be achieved with solid particles, if they have a suitable contact angle (Figure 13.6). An example is provided by the partially solid fat globules in whipped cream, which completely coat the air bubbles, and also form a network throughout the system (see Section 13.6.5).

Many complex systems contain at least some solid particles that act in this manner (being small and fairly hydrophilic). Bubble shrinkage occurs until the adsorbed solid particles touch each other. Then a small but stable bubble remains. This is presumably the cause of many undesirable persistent foams. Another example is the gas cells in bread dough [76]. They show extensive Ostwald ripening, and the number of visible cells in the final product is less than 1% of those originally present. This does not mean that all the others have disappeared. In fact, many tiny cells remain, presumably stabilized by solid particles. These cells are not visible but scatter light sufficiently to give bread crumb its white appearance.

It should be mentioned that Ostwald ripening can be prevented by a yield stress in the aqueous phase, but it would need to be high, about 10^4 Pa. An example is chocolate containing air bubbles.

13.7.2.2 Drainage

As mentioned in Section 13.2.6, immobilization of the a/w interface by means of a γ-gradient is essential to prevent almost instantaneous drainage (Figure 13.9c and 13.9d). The maximum height that a vertical film (lamella) between two bubbles can have while preventing motion of the film surfaces, is given by

$$H_{max} = \frac{2\Delta\gamma}{\rho g \delta} \tag{13.32}$$

The maximum value that $\Delta\gamma$ (between top and bottom of a vertical film) can assume equals the surface pressure Π, which would be about 0.03 N m^{-1}. For an aqueous film of thickness $\delta = 0.1$ mm, H_{max} would be 6 cm, far more than needed in food foams (6 cm is, indeed, about the height of the largest foam bubbles floating on a detergent solution).

The drainage time of a single vertical film with immobilized surfaces is given by

$$t(\delta) \approx \frac{6\eta H}{\rho g \delta^2} \tag{13.33}$$

where $t(\delta)$ is the time needed for the film to drain to a given thickness δ. For a water film of 1 mm height, only 6 s of drainage would be required to achieve a thickness of 10 μm. However, the

drainage rate diminishes with decreasing thickness, and it would take 17 days of drainage to achieve $\delta = 20$ nm. The latter is the approximate thickness at which van der Waals attraction forces between the two film surfaces come into play.

Predicting the drainage rate in a real foam is far more difficult, and accurate calculations cannot be made. Equation 13.33 will serve to provide approximate (order of magnitude) values. Drainage can, of course, be slowed down considerably by increasing the viscosity. For this purpose, viscosity should be measured at fairly low shear stress. A yield stress of about $gH\rho_{\text{water}}$ (where H is the height of the foam layer) will also arrest drainage.

13.7.2.3 Coalescence

This occurs when a film between bubbles ruptures, but the mechanism differs with circumstances. Three main cases can be distinguished:

1. *Thick films*. This refers to films thick enough so that colloidal interaction between the two surfaces is negligible. In this situation the Gibbs stabilizing mechanism is essential (Section 13.2.6, especially Figure 13.9e). Film rupture, and thereby bubble coalescence, will occur only when surfactant concentration is very low. If a film is extensively stretched, as will always occur during beating, rupture occurs more readily. Indeed, an optimum whipping speed for foam formation is observed (Figure 13.34b), that is, one that achieves greatest air incorporation.
2. *Thin films*. This concerns films thin enough for colloidal interactions to become important. The considerations given in Section 13.6.4 roughly apply (see especially Figure 13.30) and in the absence of strong colloidal repulsion keeping the film thickness relatively large, rupture of the film may readily occur. However, it may take a long time before the film has drained to small thickness. On the other hand, water may evaporate from the film, especially at the top of a foam. Hence, film rupture will especially occur at the top of a foam, leading to a decrease in foam height. Compared to emulsions, foams are far more unstable against coalescence. γ is large (more stable); the films between bubbles are "permanent" (less stable); film area is very large (less stable); moreover, far fewer films have to rupture for coalescence to become significant (see Table 13.7). Again, proteins may yield the most stable films, especially if they form thick adsorbed layers.
3. *Films containing extraneous particles*. It is often observed that the presence of extraneous particles, especially lipids, is very detrimental to foam stability. Such particles can cause rupture of relatively thick films, and several mechanisms have been postulated [28]. Presumably, spreading of oil over an a/w surface of the film plays a dominant role. Protein-covered oil droplets have a hydrophilic surface layer, and thus cannot spread oil over the a/w surface. However, if it concerns fat globules, that is, oil droplets containing triacylglycerol crystals, oil can readily reach the a/w surface; cf. the role of such crystals in partial coalescence (Section 13.6.5). Especially large fat globules are quite effective foam breakers. It is well known, for instance, that traces of lipstick are detrimental to beer foam stability. Another example is given by skim milk, which contains less than 0.05% fat and only very small globules, that can be made to foam very much better than whole milk.

In relation to this, the number concentration of extraneous particles should be considered. A typical food foam contains, say, 10^{12} lamellae per m^3 of liquid phase. Presumably, 10^{12} particles per m^3 would thus suffice to cause substantial bubble coalescence, provided these particles can induce film rupture. The larger milk fat globules, say, 6 μm in diameter, when containing both liquid and solid fat, would be suitable. 10^{12} of these per m^3, will correspond to about 0.01% of fat. A very small amount can thus induce significant coalescence.

In a typical whipping cream, the number of partially solid fat globules is very large, at least 10^{16} m^{-3}. Many of these globules would be able to induce film rupture. However, their large concentration causes almost simultaneous adsorption of many globules very close to each other. Spreading of liquid oil over any distance then is not possible, film rupture will rarely occur, and a stable and rather firm foam results. However, if whipping goes on, the fat globules undergo extensive partial coalescence, large clumps are formed, and eventually their number becomes so small that film rupture can occur. In other words, overwhipping destroys the foam made at an earlier stage. When churning cream to obtain butter granules, that is, large clumps of fat globules, this occurs on purpose.

FREQUENTLY USED SYMBOLS

A	(Specific) surface area	(m^{-1}, m^2)
	Hamaker constant	(J)
a	Thermodynamic activity	(mole fraction)
	Acceleration	($m\ s^{-2}$)
B	Permeability	(m^2)
c	Concentration	($kg\ m^{-3}$; $mol\ m^{-3}$; $mol\ L^{-1}$)
D	Diffusion coefficient	($m^2\ s^{-1}$)
	Fractal dimensionality	(–)
d	Particle diameter	(m)
E_{SD}	Surface dilational modulus	($N\ m^{-1}$)
F	(Gibbs) free energy	(J; $J\ mol^{-1}$)
f	Force	(N)
G	Elastic shear modulus	(Pa)
g	Acceleration due to gravity	($9.81\ m\ s^{-2}$)
H	Height	(m)
	Enthalpy	(J; $J\ mol^{-1}$)
h	Interparticle distance	(m)
I	Ionic strength	($mol\ L^{-1}$)
k	Boltzmann constant	($1.38 \times 10^{-23}\ J\ K^{-1}$)
l	Distance, length	(m)
m	Concentration	($mol\ L^{-1}$)
N	(Total) number concentration	(m^{-3})
n_i	Number of particles in class i	(m^{-3})
p	Pressure	(Pa)
p_L	Laplace pressure	(Pa)
Q	Volume flow rate	($m^3\ s^{-1}$)
R	Universal gas constant	($8.314\ J\ mol^{-1}\ K^{-1}$)
	Radius of aggregate (floc)	(m)
R_{cr}	Critical radius	(m)
R_g	Radius of gyration	(m)
r	Particle radius	(m)
S	Entropy	($J\ K^{-1}$; $J\ mol^{-1}\ K^{-1}$)
s	Solubility of gas	($mol\ m^{-3}\ Pa^{-1}$)
T	(Absolute) temperature	(K)
t	Time	(s)
$t_{0.5}$	Halving time	(s)
V	Interaction free energy	(J)
v	Velocity	($m\ s^{-1}$)
v_S	Stokes velocity of particle	($m\ s^{-1}$)

∇v	Velocity gradient; shear rate	(s^{-1})
W	Stability ratio	(–)
x	Distance	(m)
z	Valence	(–)

Greek Symbols

Γ	Surface excess (load)	(mol m^{-2}, kg m^{-2})
γ	Surface/interfacial tension	(N m^{-1})
δ	Layer (film) thickness	(m)
ε	Strain (relative deformation)	(–)
ε_{fr}	Strain at fracture	(–)
θ	Contact angle	(rad)
κ	Reciprocal Debye length	(m^{-1})
η	Viscosity	(Pa s)
η_a	Apparent viscosity	(Pa s)
Π	Surface pressure	(N m^{-1})
Π_{osm}	Osmotic pressure	(Pa)
ρ	Mass density	(kg m^{-3})
σ	Stress	(Pa)
σ_{fr}	Fracture stress	(Pa)
σ_y	Yield stress	(Pa)
ϕ	Volume fraction	(–)
ψ	Fraction solid	(–)
	Electric potential	(V)

Subscripts

A Air
C Continuous phase
D Dispersed phase
O Oil
S Solid
W Water (aqueous phase)

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14 Physical and Chemical Interactions of Components in Food Systems

Zdzisław E. Sikorski, Jan Pokorny, and Srinivasan Damodaran

CONTENTS

14.1 INTRODUCTION

The main components of most foods are water, proteins, saccharides, lipids, fiber, organic acids, and mineral compounds in various proportions. Foods also contain many other minor constituents and additives. These compounds constitute the structural elements of the tissues, serve as energy reserves, and have numerous biochemical functions in living plants and animals. Most food constituents are chemically reactive or at least contain various reactive groups (Table 14.1). In many instances, some of these groups are unavailable for reaction/interaction due to physical barriers. The interactions in foods are controlled by the chemical character of these groups, compartmentation in the internal architecture of the tissues, and by the environmental conditions, such as temperature, pH, ionic strength and type of ions (e.g., polyvalent cations), water activity, oxidation/reduction potential, and viscosity of the fluids. All these factors undergo alterations during postharvest/postmortem handling and storage of the raw materials and as a consequence of processing.

Physical interactions between various constituents often lead to changes in physical and sensory attributes of foods. Some examples are given below:

- Rupturing of the cell membranes in fish muscle by large ice crystals formed in the intracellular spaces during slow freezing; this leads to extensive drip loss during thawing of the fillets; similar processes also occur in frozen fruits and vegetables.
- Selective cell breakage under controlled conditions of milling results in disruption of the endosperm cells of the wheat grain, but leaves the starch granules and the aleurone layer associated with the bran mostly undamaged.
- Cell disruption in fruits caused by different physiological processes accompanied by activation of pectinases, polyphenol oxidase, cellulases, and invertase; these enzymic processes facilitate the development of typical sensory properties of the ripened fruit.

High hydrostatic pressure of the order of 100–700 MPa promotes several interactions in food materials, which impart quality changes in foods. Examples of some of these changes are coagulation of egg white and yolk, opacity of raw fish muscle, gelling and coagulation of fish sarcoplasmic

TABLE 14.1
Reactive Groups of Food Components

Reactive Groups	Source
$-SH$, $-S-S-$	Proteins, peptides, and amino acids
$-NH_2$, $-NH-C(=NH)NH_2$	Proteins, amino acids, and other amine containing compounds
$-OH$, $-CHO$, $R_2C=O$	Proteins, carbohydrates, and low molecular weight carbonyls
$^1O_2$, $^{\bullet}O_2^-$, $^{\bullet}OH$, H_2O_2, $RO^{\bullet}$, $ROO^{\bullet}$, ROOH, $ArO^{\bullet}$, $ArOO^{\bullet}$	Products of lipid oxidation
$-COOH$, $-O-SO_3H$, $-O-PO_3H_2$	Proteins, pectins, and other polysaccharides
$-CH=CH-$, $-CH=CH-CH_2-CH=CH-$	Unsaturated lipids
$NO^{\bullet}$, $NO_2^{\bullet}$, $O=N-OOH$, $O=N-OO^-$	Additives

proteins, denaturation and insolubilization of β-lactoglobulin and to a lesser extent α-lactalbumin, disruption of casein micelles, modifications of the sarcomere structure in meat, depolymerization of isolated myofibrillar proteins, tenderization of meat, inactivation of several endogenous fish enzymes (including those involved in *postmortem* degradation of high-energy phosphates), acceleration of lipid oxidation in fish muscle, hydrolysis and gelatinization of starch, and crystallization of milk fat. The extent of changes depends on the level of the applied high pressure and on other processing parameters, mainly temperature, pH, and time of treatment [1–3].

Physicochemical interactions between food components are responsible for the formation of dispersions, that is, emulsions and foams, and for the rheological properties of various products. They affect the sensory quality and processing attributes of food materials, such as flow properties and resistance to shear. Chemical reactions between food components play a crucial role in the development of both desirable and undesirable sensory and nutritional properties of foods. Therefore, a comprehensive understanding of the various physicochemical interactions between the components is necessary for managing and improving food quality. Major reactions that proteins, carbohydrates, and lipids undergo during the processing and handling of foods are summarized in Figure 14.1 through 14.3.

14.2 TYPES OF MICROSTRUCTURES IN FOODS

Chemical and physical interactions between food constituents, such as water, proteins, lipids, saccharides, fiber, mineral compounds, and dissolved and dispersed gases, produce various macroscopic structures in natural and formulated foods. Some of the common structural forms are droplets or globules, fibrils or strands, sheets and films, vesicles, micelles, granules, crystals, and bubbles [4]. These structures are stabilized in the predominantly aqueous food systems by covalent bonds, ionic forces, hydrogen bonds, and hydrophobic interactions. The size and shape of such structures and their stability, distribution, and interactions, being affected by the biochemical state of the raw materials and by the processing variables, have a large impact on many quality attributes, especially on the texture and appearance of foods.

14.2.1 Water Droplets, Fat Globules, and Polymorphic Phases of Lipids

Water droplets are present in water-in-oil emulsion type products, for example, butter, margarine, and table spreads. In products such as margarine and butter the water content is about 20%. Spreads, on the other hand, contain less fat (between 20% and 70%) and more water, so that their calorie content is lower than that of margarine. In butter the diameter of the water droplets is below 10 μm, and in spreads it should be kept below 5 μm to reduce microbial growth. The continuous phase of the margarine emulsion is made up of an interconnected network of liquid oil, fat crystals, and sheet-like fat crystal aggregates. In butter the globules that remain intact after churning form a network in a matrix of free fat. The core of liquid fat of these globules is surrounded by fat crystals. The softening temperature of butter depends on the proportion of fluid fat to fat crystals. The differences in the physical state of the continuous phase of butter and margarine are responsible for differences in their physical properties, such as spreadability.

Lipid globules exist as a dispersed phase in an aqueous solution in several food products, such as milk, dairy products, mayonnaise, salad dressings, and comminuted meat and fish products (Figure 14.4). The fat globules (0.5–10 μm in diameter) in fresh milk are separated from each other and from the aqueous medium by a 10-nm-thick biomembrane derived from the mammary gland. The surface of these membranes is covered by adsorbed lipoprotein particles. Processing induces changes in the size distribution of fat globules and thereby affects the creaming stability of the globules in milk. For instance, in ultrafiltered milk the globules are larger (1–18 μm in diameter) as

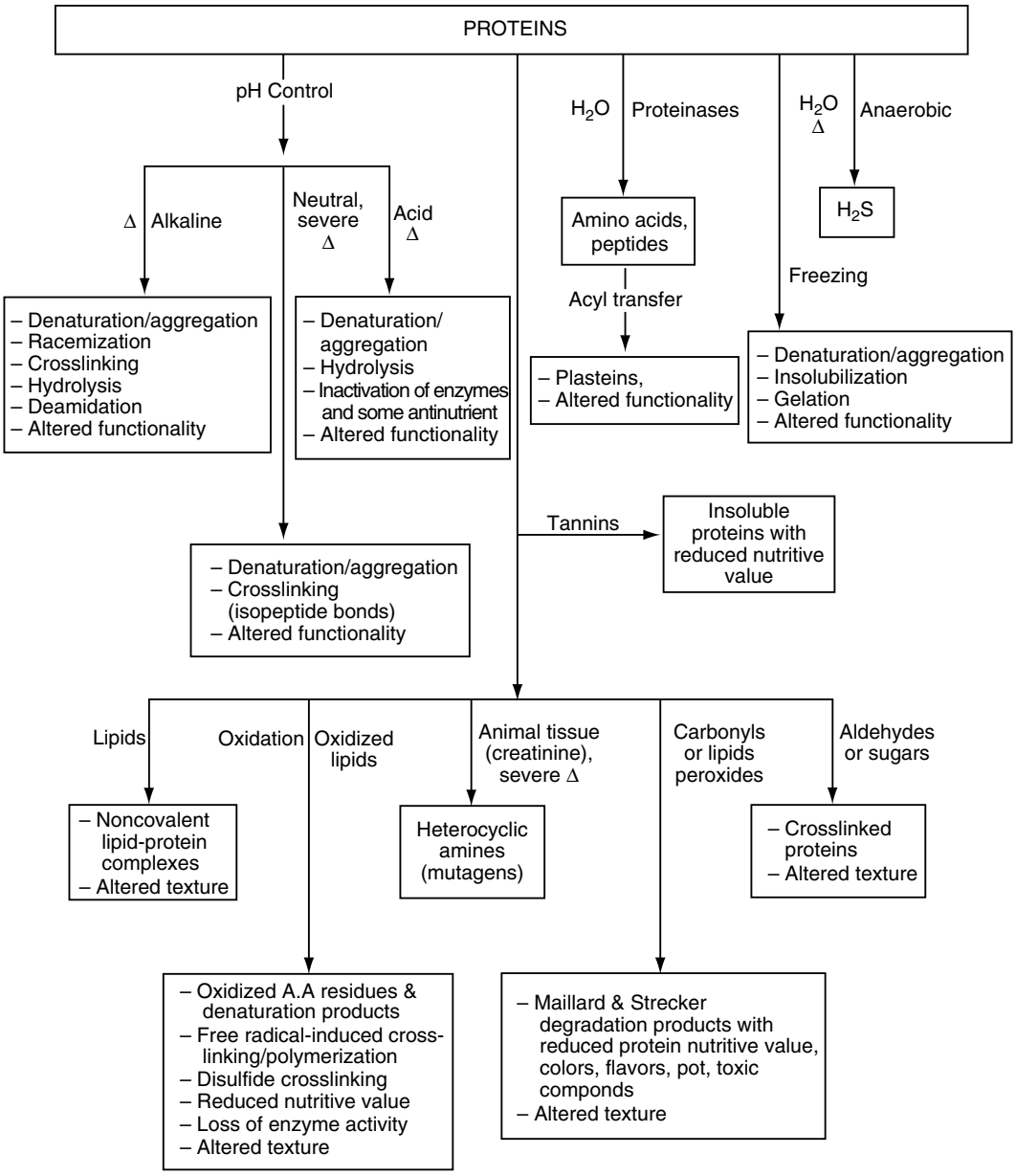

FIGURE 14.1 Major reactions that proteins can undergo during the processing and handling of foods. (From P. Taoukis, T. P. Labuza (1996). In: *Food Chemistry*, 3rd edn. (O. Fennema, ed.), Marcel Dekker, New York., p. 1015).

a result of coalescence of droplets caused by the shearing action on the membranes (Figure 14.5). In whipped cream and ice cream, the fat globules are adsorbed at the aqueous–air interface of the dispersed air bubbles. In fresh cheese, the milk fat globules are surrounded by a protein shell and distributed in a protein network (Figure 14.6). In mayonnaise, the closely packed oil droplets form a honeycomb structure. This structure is stabilized within the aqueous continuous phase by the egg yolk phospholipids adsorbed at the oil–aqueous interface and probably also by lipid crystals at the interface [5].

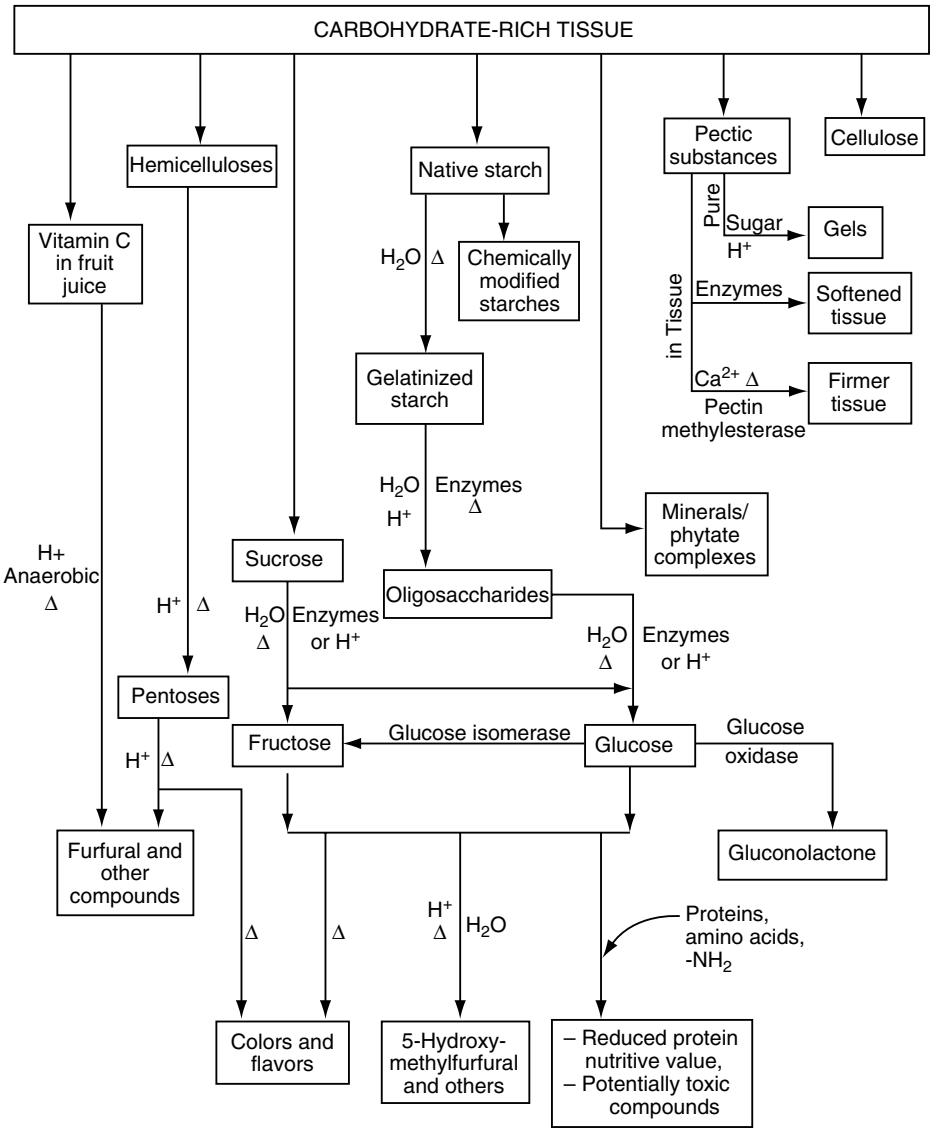

FIGURE 14.2 Major reactions that carbohydrates can undergo during the processing and handling of foods. (From P. Taoukis, T. P. Labuza (1996). In: *Food Chemistry*, 3rd edn. (O. Fennema, ed.), Marcel Dekker, New York., p. 1016).

The rheological properties of various milk gels and cheeses depend on the size and number of the fat droplets and the nature of the stabilizing agent. For instance, low molecular weight surfactants make the surface of the fat droplets smooth and noninteractive. As a result, the gels containing such droplets are weak and have high meltability. In contrast, milk gels containing fat globules stabilized by whey proteins are strong and have low meltability. This is due to protein–protein cross-linking between adsorbed layers of whey protein around the fat globules.

Amphiphilic lipids assemble in aqueous environments as micelles or lamellar phases (see Chapter 4). Micelles are formed when the concentration of lipids and the temperature are above the respective critical values. Depending on the properties of the lipids two types of micelles can occur. The hexagonal micelles may have their polar heads protruding to the surface and the hydrocarbon chains directed axially toward the center of the cylinder, or the polar groups point to the axis forming

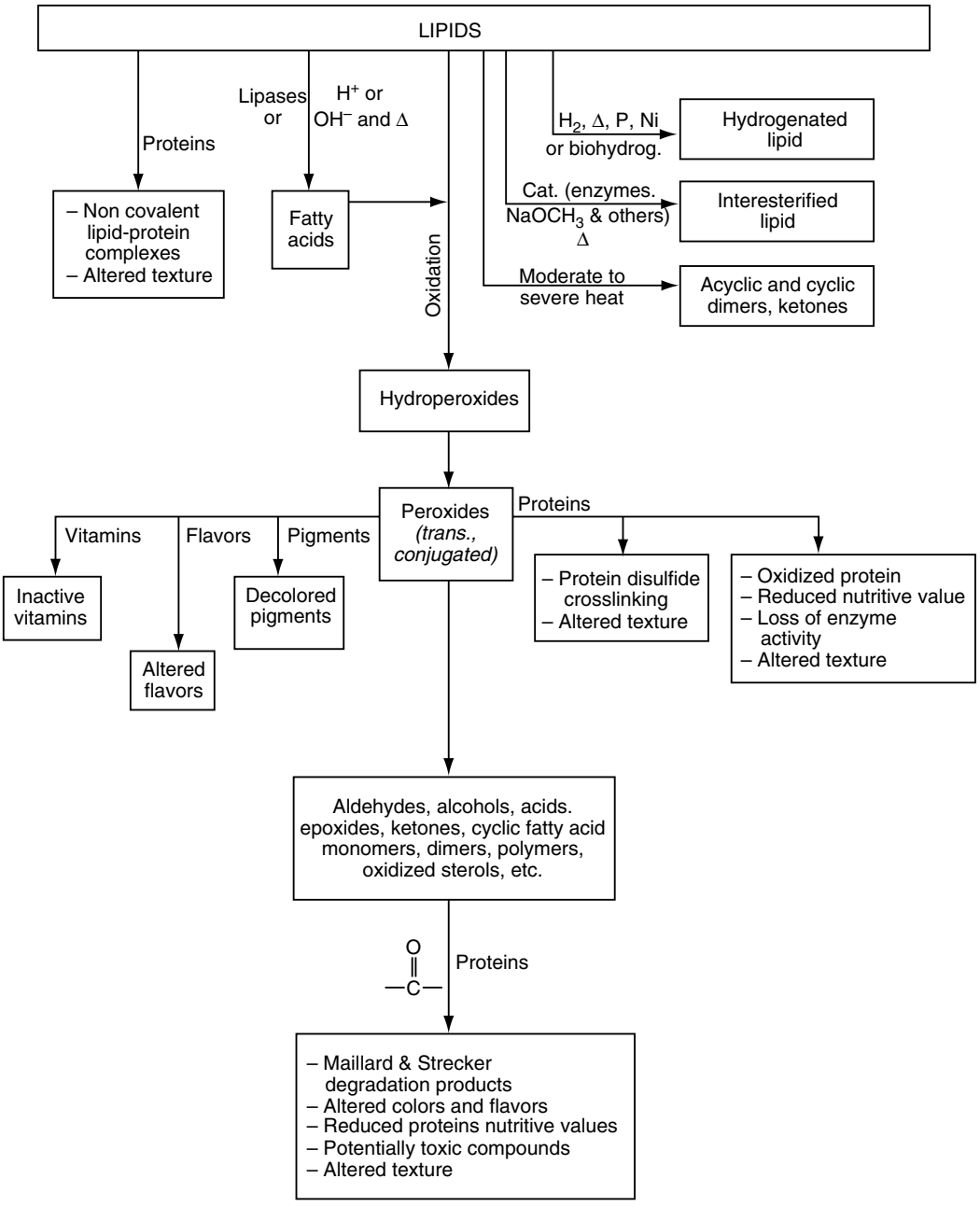

FIGURE 14.3 Major reactions that lipids can undergo during the processing and handling of foods. [a]Oxidation as catalyzed by enzymes, metals, myoglobin, chlorophyll, and/or irradiation. (From P. Taoukis, T. P. Labuza (1996). In: *Food Chemistry*, 3rd edn. (O. Fennema, ed.), Marcel Dekker, New York., p. 1017).

a water channel inside, with the hydrophobic tails oriented outside (reverse micelles). Micelles have various shapes, which are affected by the surface area of the polar groups and the volume and length of the hydrophobic chains. The lamellar liquid crystalline phases are self-assembled by stacking of stretched lipid bilayers separated by water lamellae. In wheat flour lipids, a transition from the micellar to the lamellar phase takes place on hydration [6].

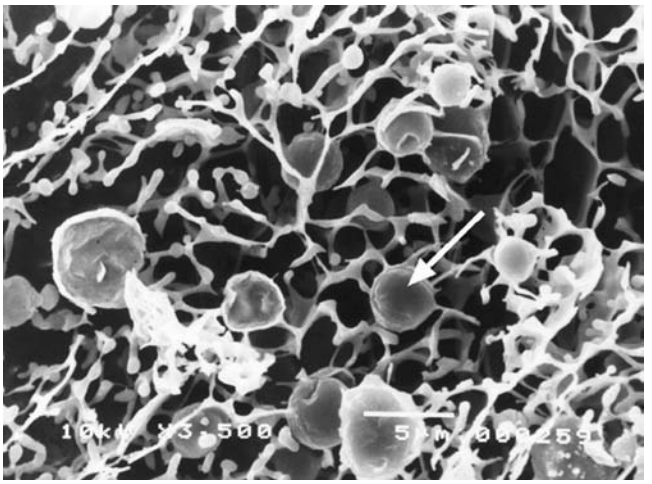

FIGURE 14.4 Cryo-SEM micrograph of fresh milk. Fat globule (arrow). (Courtesy of CRC Press: M. A. Lluch, I. Hernando, and I. Pérez-Munuera (2003). In: *Chemical and Functional Properties of Food Lipids* (Z. E. Sikorski and A, Kołakowska, eds.), CRC Press, Boca Raton, London, New York, Washington, DC, pp. 9–28, Figure 2.6).

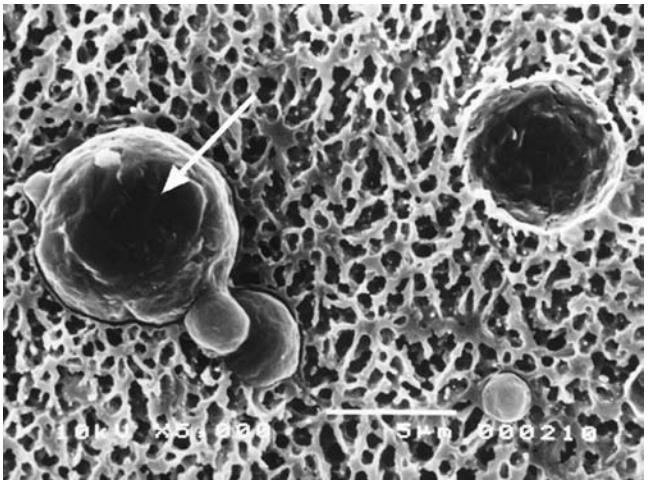

FIGURE 14.5 Cryo-SEM micrograph of ultrafiltered milk. Fat globules (arrow). (Courtesy of CRC Press: M. A. Lluch, I. Hernando, and I. Pérez-Munuera (2003). In: *Chemical and Functional Properties of Food Lipids* (Z. E. Sikorski and A., Kołakowska, eds.), CRC Press, Boca Raton, London, New York, Washington, DC, pp. 9–28, Figure 2.7).

14.2.2 Fibrils, Sheets, and Protein Micelles

The microstructure of several natural and fabricated food products consists of protein fibrils, filaments, and sheets. A good example is meat products (see Chapter 16). Similarly, the microstructure of wheat gluten is composed of protein fibrils/strands with starch granules adhering to the protein matrix (Figure 14.7). During preparation of bread dough, hydration and swelling of starch and gluten particles facilitate cross-linking between protein polymers, which results in the formation of a viscoelastic network [7]. In general, interactions between proteins and polysaccharide fibrils/strands are responsible for gel-like texture in many food products.

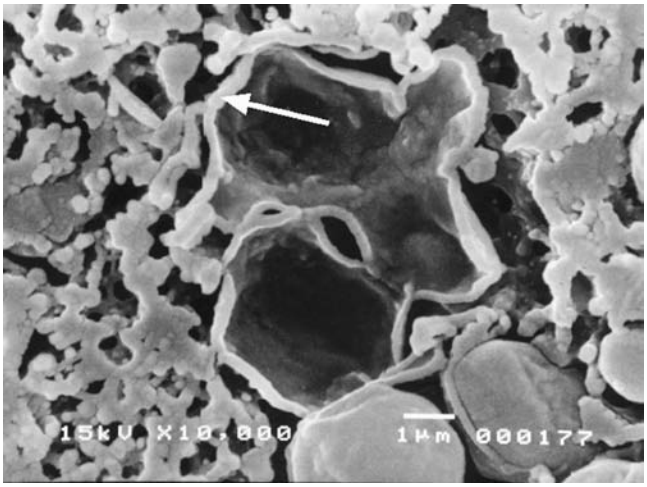

FIGURE 14.6 Cryo-SEM micrograph of fresh cheese. Protein shell of the fat globule membrane (arrow). (Courtesy of CRC Press: M. A. Lluch, I. Hernando, and I Pérez-Munuera (2003). In: *Chemical and Functional Properties of Food Lipids* (Z. E. Sikorski and A., Kołakowska, eds.), CRC Press, Boca Raton, London, New York, Washington, DC, pp. 9–28, Figure 2.10).

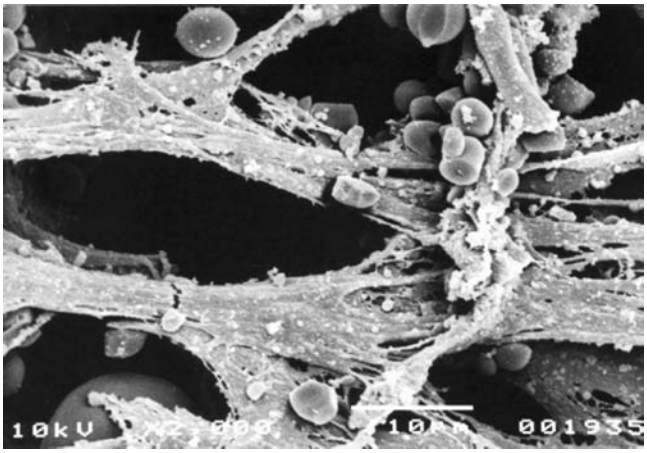

FIGURE 14.7 Fibrils and strands in wheat gluten. Small starch granules adhering to the protein matrix are clearly visible. (Courtesy of Professor Józef Fornal.)

Sheets composed of nonstarchy polysaccharides, such as pectins, cellulose, and hemicellulose, form a sponge-like structure in the seed hulls of cereal grains (Figure 14.8) that protect the seeds against external factors. The sponge-like structure is also responsible for the texture of the apple tissue (Figure 14.9). Polydisperse protein micelles are formed by the caseins in milk.

14.2.3 Granules and Crystals

Starch is present in many starchy raw materials and in processed foods in the form of granules of spherical, oval, polygonal, elongated, or other shapes. The diameter of the granules differs depending on the botanical source of the material; it is 0.5–2 μm for amaranth starch, 5–20 μm for maize starch, and 15–75 μm for potato starch (see also Chapter 3). The granules are composed of amylose and

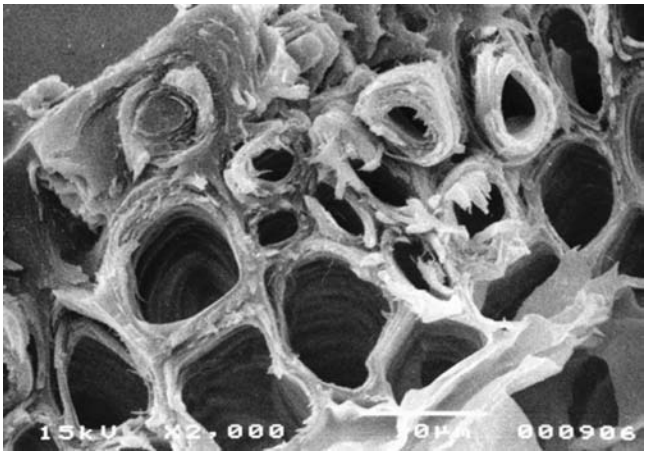

FIGURE 14.8 Cells of barley kernel seed coat. (Courtesy of Professor Józef Fornal).

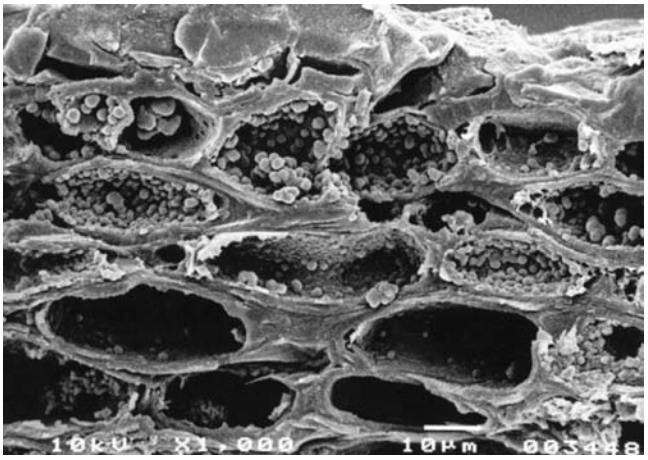

FIGURE 14.9 Sponge-like structure of apple tissue. The outer layer (waxy substances) protects the fruit against water evaporation, minute starch granules are the main components of the cells below, and the cell walls are cemented together by pectic substances. (Courtesy of Professor Józef Fornal.)

amylopectin and also contain 1–2% of lipids, proteins, and mineral components. The lipids present in natural starch occur as inclusion complexes inside the polysaccharide helices, as surface inclusion complexes, and within the capillaries between starch granules [8]. In the internal structure of the starch granules alternating crystalline and amorphous lamellae are arranged concentrically around the hilum. In presence of water the microstructure of the granules is altered (Figure 14.10). Initially, at comparatively low water content, a layer of amorphous amylose from the inside is displaced to the surface of the granules due to swelling and small pores appear on the surface caused by the activity of enzymes. At water content above 30% and at sufficiently high temperature, gelatinization occurs [9].

Starch particles with a uniform size distribution of 1–2 μm act as fat mimetic by imparting "lubricity" or fatty sensation to the food. This is particularly the case in food gels made from plant sources that contain much starch. Ca-pectinate particles of about 40 μm in diameter also possess this property.

Depending on the size distribution, protein particles also contribute to the textural properties of many creamy and gelatinous processed foods. In this respect, egg white and milk proteins are

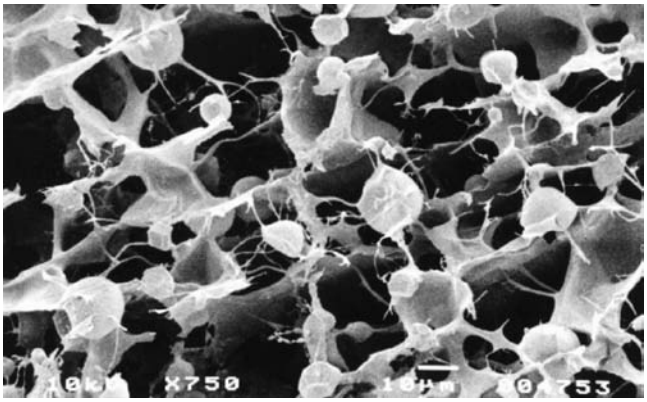

FIGURE 14.10 Microstructure of tapioca starch paste (8%) cooked at 64°C for 15 min. Gelatinisation of starch granules can be seen—hydrated granules forming a network with solubilized amylose that leaked from the swollen granules. (Courtesy of Dr. Wioletta Blaszczak.)

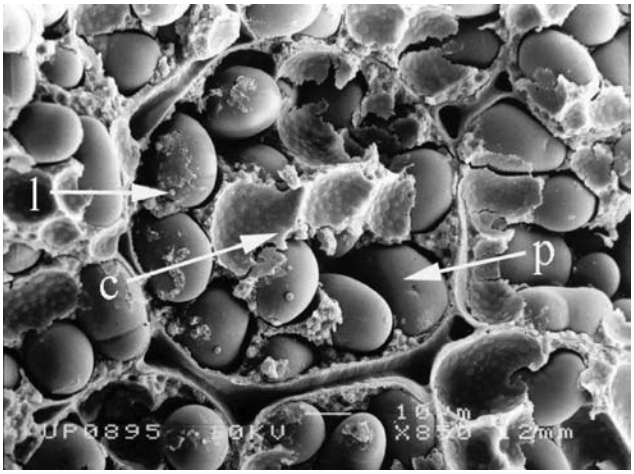

FIGURE 14.11 SEM micrograph of soybean cotyledon cells. Protein bodies (p), lipid bodies (l), cytoplasmic network (c). (Courtesy of CRC: From M. A. Lluch et al. (2001). Proteins in food structures. In: *Chemical and Functional Properties of Food Proteins* (Z. E. Sikorski, ed.), CRC Press, Boca Raton, London, New York, Washington, DC, pp. 13–33, Figure 2.7.)

the proteins of choice because of their propensity to form aggregated particles under appropriate processing conditions. The dimensions of the particles have a significant effect on the rheological properties of the material. Very small ones, such as casein micelles (~0.1 μm in diameter), impart gelatinous and lubricity characteristics, while those of the order of 2 μm in diameter exhibit creamy and rich texture; larger particles (>50 μm) are responsible for powdery and gritty perception [4].

Almost all seeds contain protein granules, also known as protein bodies or aleurone grains. These bodies, generally spherical, differ in size, depending on the species and variety of the cereal or legume. The average diameter is about 2 μm in corn, 0.5–3 μm in sorghum, 1–5 μm in broad beans, 2–10 μm in peanuts, and 2–20 μm in soybean. The protein bodies of soybeans are nestled within a sponge-like cytoplasmic protein network [10] (Figure 14.11).

Fat crystals are common in various dairy products, in chocolate and in shortenings (Figure 14.12). Triacylglycerols (TGs) exist in several crystalline forms, the principal ones being α, β', and β (see Chapter 4). They differ in stability and melting temperatures. The TGs containing the same

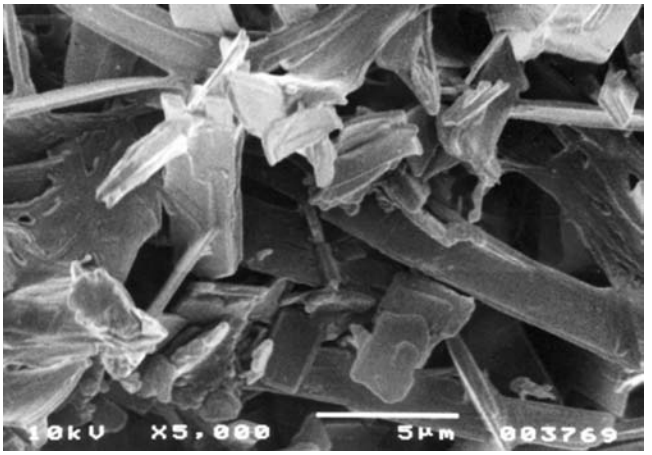

FIGURE 14.12 Fat crystals in spread. (Courtesy of Professor Józef Fornal.)

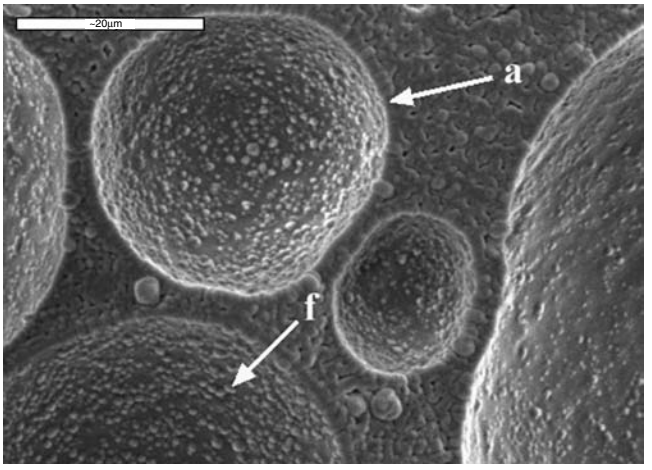

FIGURE 14.13 Cryo-SEM micrograph of ice cream. Air cell (a); fat globule at the interface (f). (Courtesy of CRC: From M. A. Lluch, I. Hernando, and I. Pérez-Munuera (2003). Lipids in food structures. In: *Chemical and Functional Properties of Food Lipids* (Z. E. Sikorski and A. Kołakowska, eds.), CRC Press, Boca Raton, London, New York, Washington, DC, pp. 9–28, Figure 2.8.)

fatty acyl chain at all the three positions are most stable in the β form. TGs with mixed acyl chains crystallize as either β' or β crystals. The TGs of cottonseed, palm, rapeseed, and herring oils, as well as in milk fat and tallow mainly assume the β' form, while the TGs of soybean, sunflower, peanut, olive, corn, canola, coconut oil, cocoa butter, and lard have a tendency to form stable β crystals. In margarine and in shortenings, the β' crystals are desirable. Their size is about 1 µm long with needle-like shape; they also appear in the form of flat platelets. These structures can accommodate a large number of small air bubbles, which impart plasticity and creaminess to the products. Transformations of the polymorphic forms of cocoa butter are thought to be responsible for the development of the undesirable appearance of white or gray powdery surface deposits and loss of gloss of stored chocolate, known as bloom. Large spherulites with random arrangement of crystals may be responsible for sandy texture of various foods.

Air bubbles are typical components of ice cream structure (Figure 14.13), whipped cream, bread, and beer foam. The gas bubbles are dispersed in a solid matrix, for example, in bread or cake, or

in a fluid such as in whipped cream where a protein film stabilizes them. The size distribution and the stability of these dispersed air bubbles play a crucial role in the sensory attributes, especially the texture, of these products.

14.3 CHEMICAL INTERACTIONS OF FOOD COMPONENTS

14.3.1 Introduction

The chemical interactions of food components under conditions of storage and processing comprise predominantly the Maillard reaction, caramelization of sugars, other pyrolytic changes, interactions of quinones with amines and amino acids, various oxidation processes, and reactions of proteins in alkaline conditions. The results of these interactions may be desirable, for example, browning of the crust of bread, in coffee roasting, or onion frying. In other cases, they may be detrimental for the food quality, for example, in stored condensed milk or dry vegetables. In many reactions flavor substances are produced, which may or may not be desirable, depending on the particular food. The nutritional value and safety of foods may decrease due to loss of valuable components or formation of some mutagenic and carcinogenic compounds. However, the gain in sensory quality is most often much higher than the small loss in biological value.

14.3.2 The Maillard Reaction

14.3.2.1 Nomenclature and Substrates

The Maillard reactions are sometimes called nonenzymic browning because the end products are brown colored melanoidins. Nonenzymic browning is also caused by caramelization of sugars, interactions of quinones with amines and amino acids, or interactions of oxidized lipids with protein. Nonenzymic browning is easily distinguished from enzymic browning, which is related to enzymic oxidation of food polyphenols.

The most important saccharides participating in the Maillard reactions are glucose and fructose, while in meat it may be ribose. Among disaccharides, lactose is an important browning precursor in dairy products as is maltose in cereal products, such as malt. Sucrose is easily cleaved into glucose and fructose, especially on heating. Therefore, it can participate in nonenzymic browning quite easily. Sugars bound as glycosides, for example, in glycoproteins, glycolipids, and in heteroglycosides are less reactive, but the aglycones may be released during heating to yield free reducing saccharides.

The other reaction partners are proteins, peptides, amino acids, and other amine compounds. The reactive group of proteins is mainly the ε-amino group of the lysine residue. The end amino groups of peptidic chains may also participate in the reaction, but their concentration in proteins is at least several times lower than that of lysine. The guanidyl group of arginine or the thiol group of cysteine can also participate in Maillard browning. In some food products, especially in cheeses and fish, biogenic amines are also precursors of brown products. Ammonium hydroxide and ammonium salts are reactive as well as amines.

Aldehydes and other carbonyls also participate in the browning reaction. They may originate from sugar derivatives, such as ascorbic acid, but they may also be produced from other precursors, predominantly oxidized lipids.

14.3.2.2 The Mechanism and Products of Reaction

The Maillard browning reaction proceeds in three stages:

1. Reaction of an amine with a reducing sugar with the formation of glycosyl amine, followed by the Amadori rearrangement (Figure 14.14).

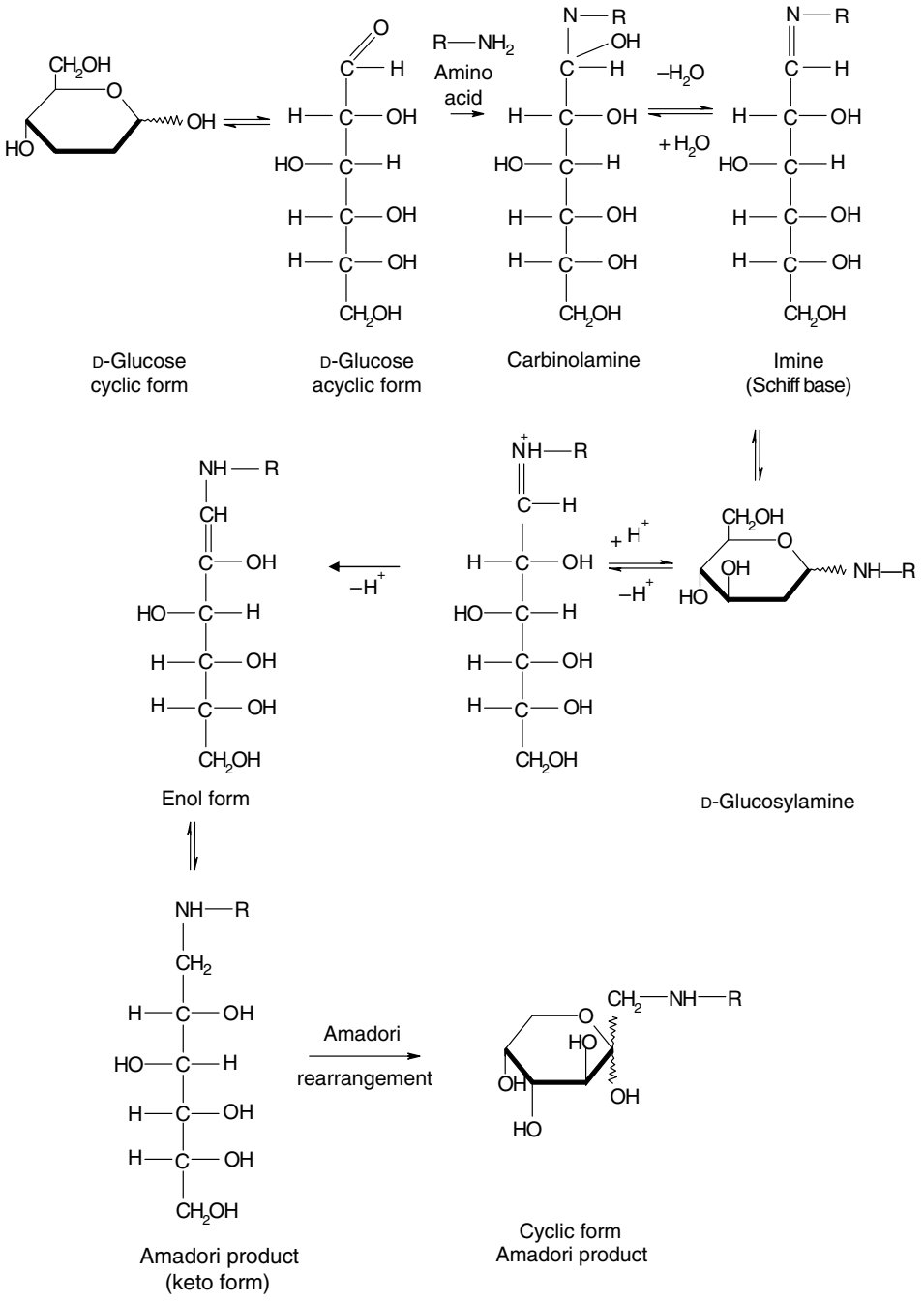

FIGURE 14.14 Formation of glucosylamine and Amadori rearrangement.

2. Dehydration of the intermediary products, fragmentation of the saccharidic moiety, and the Strecker degradation of the products (Figure 14.15).
3. Reactions of intermediary products resulting in the formation of heterocyclic flavor compounds, that is, high molecular weight brown pigments, which are responsible for the typical flavor of brown products (Figure 14.16).

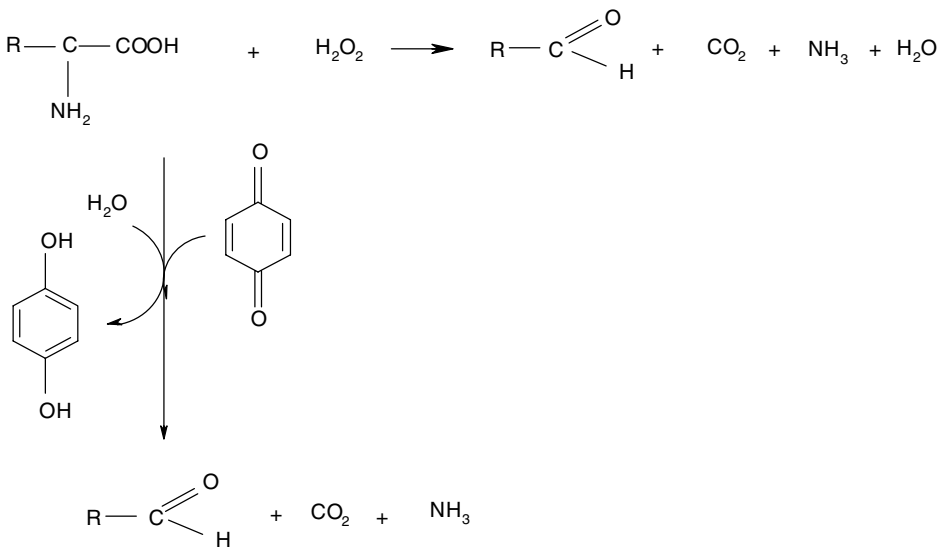

FIGURE 14.15 Strecker degradation of amino acids with oxidizing agents.

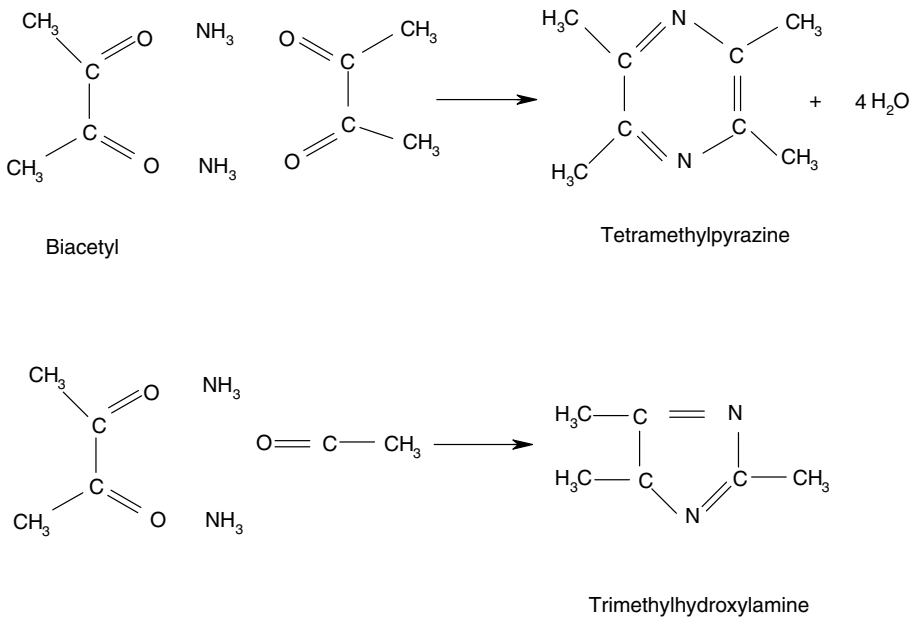

FIGURE 14.16 Formation of heterocyclic flavor compounds.

The Maillard reaction starts with the addition of a nonprotonized amine group to the electrophilic carbonyl carbon of a reducing sugar (Figure 14.14). The addition product, a monotropic carbonylamine or similar compound, is dehydrated with the formation of an imine or a Schiff base (or an azomethine). The addition rate increases with increasing electronic density of the nucleophilic amine. The inductive effect of the carbonyl group substituents and steric factors are also important.

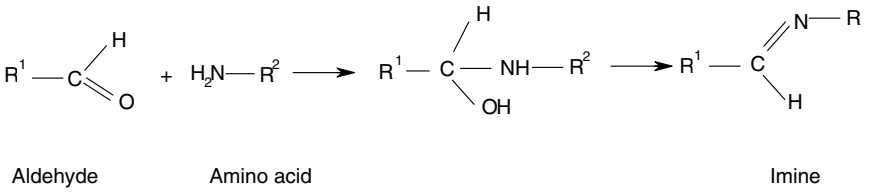

FIGURE 14.17 Reaction of carbonylic compounds with amine groups.

The reactivity of a carbonyl group decreases in the following series:

- Aldoses are more reactive with amino acids than ketoses.
- Trioses react with amino acids more rapidly than tetroses, tetroses more rapidly than pentoses, which are more reactive than hexoses and disaccharides.
- α-Dicarbonyl derivatives react with amino acids more easily than aldehydes, which are more reactive than ketones; reducing sugars are still less reactive, followed by oxo-acids.
- Acyclic form of sugars reacts more easily with amino acids than cyclic form of sugars.

The reactivity of amino compounds depends on their basicity: ammonium hydroxide and ammonium ions react with reducing sugars more easily than amines, whose reactivity decreases with their decreasing basicity.

Protonation of the carbonyl group increases its reactivity toward nucleophilic agents, while protonation of the amino group decreases the reactivity. Interactions of a carbonyl group with an amine group are shown in Figure 14.17. With decreasing pH, the concentration of protonated carbonyl group increases, but the concentration of nonprotonated amine group decreases. As a result, the reaction rate reaches a maximum in a slightly acidic medium in case of reaction with amines, while this occurs in a slightly basic medium in the case of reaction with amino acids.

The unstable Schiff's base is stabilized by consecutive reactions, for example, the reversible reaction between the carbonyl and the amine group. Glycosyl amines occur in aqueous solutions in the form of respective pyranoses or furanoses, similar to the original sugars. They also easily undergo mutarotation or are hydrolyzed into the original sugars and amine.

In the subsequent Amadori rearrangement the *N*-aldosyl amines yield ketosamines or 1-amino-1-deoxyketones, a step that is not reversible. *N*-ketosylamines are converted in the Heyns rearrangement into aldosamines or 2-amino-2-deoxyaldoses following a similar mechanism. Both reactions are acid catalyzed. Aldosamines occurring as furanoses are about ten times more reactive than the respective pyranoses. The reacting amino acid has the function of a catalyst at the same time, since its carboxyl group can donate the proton. Other carboxylic acids and phosphates also can catalyze the reaction.

Aldosyl amines derived from amino acids and other primary amines can react with another aldose molecule with the formation of dialdosyl amines. They are rearranged to diketosamines in a similar way as in the case of the Amadori rearrangement.

In the retroaldolization reaction of glycosylamines, very reactive dicarbon intermediary products are formed, for example, *N*-alkylimine of glycolaldehyde and D-erythrose. Alkylimines immediately dimerize into *N*, *N*′-dialkyldihydropyrazines, which are easily oxidized into pyrazinium salts. Imines are also hydrolyzed, forming glycol aldehyde, which is oxidized into glyoxal. Glyoxal is also produced by retroaldolization of hexos-2-uloses. Amino acids containing sulfur and aromatic groups are transformed into different heterocyclic compounds, for example, D-glucose reacts with cysteine forming 2-(D-*gluco*-1,2,3,4,5-pentahydroxypentyl)-thiazolidine-4-carboxylic acid.

All aminodeoxysugars (Figure 14.18) are strong reducing agents, more reactive than the original sugars. They mutarotate in aqueous solutions forming an equilibrium mixture of individual

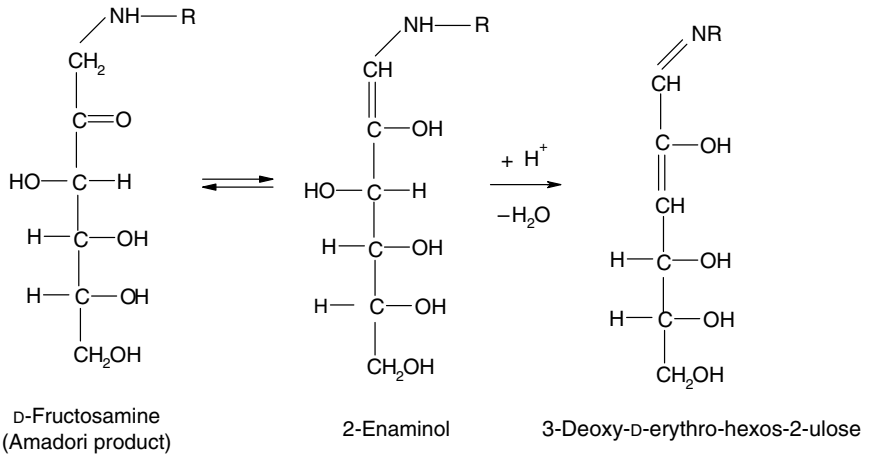

FIGURE 14.18 The 1,2-enolization of the Amadori product.

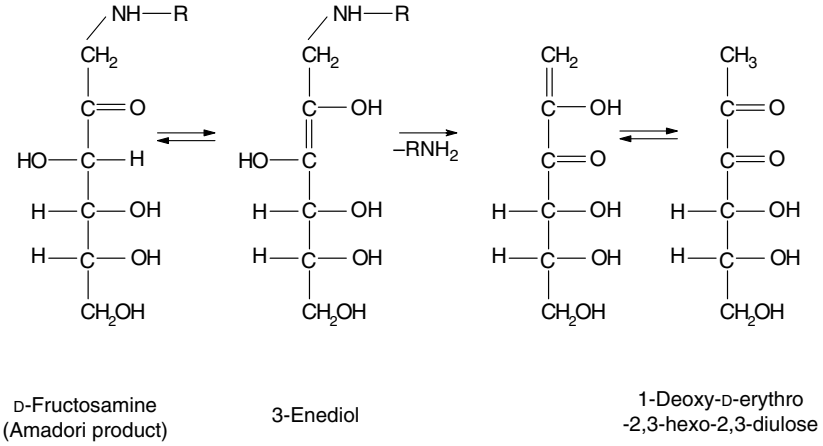

FIGURE 14.19 The 2,3-enolization of the Amadori product.

steric forms. Ketosamines (Amadori products) are relatively stable in the solid state or in neutral aqueous solutions. However, their stability is substantially lower than that of the original sugar, both in acidic and in alkaline media. The decomposition is more rapid if the compound exists in the furanose or acyclic form. The equilibrium mixtures always contain small amount of the acyclic form. Aldosamines (Heyns products) are decomposed similarly as ketosamines.

The decomposition of ketosamines begins with the 1,2-enolization. The amino analogue 1-en-1,2-diol or 1-en-1-amin-2-ol results from the degradation (Figure 14.18). The cleavage of the amino derivative 1-amino-2-deoxy-hexos-2-ulose results in the formation of 3-deoxy-D-erythro-hexo-2,3-diulose, which is further converted through dehydration into 3,4-dideoxy-D-*glycero*-hex-3-enos-2-ulose, 5-hydroxymethylfuran-2-carbaldehyde, and other compounds.

Another reaction is 2,3-enolization (Figure 14.19) with the formation of 2-ene-2,3-diol, which decomposes further to 1-deoxy-D-*erythro*-hexo-2,3-diulose. These products are similar to those formed during the degradation of sugars in the absence of amine derivatives, that is, during their caramelization, but amino acids and other amine derivatives catalyze the sugar degradation. The reaction proceeds readily at ambient temperature and in a slightly acidic medium (pH 4–7), common in foods. Although, mainly, 3- and 4-deoxy derivatives are formed in the absence of amino compounds, the

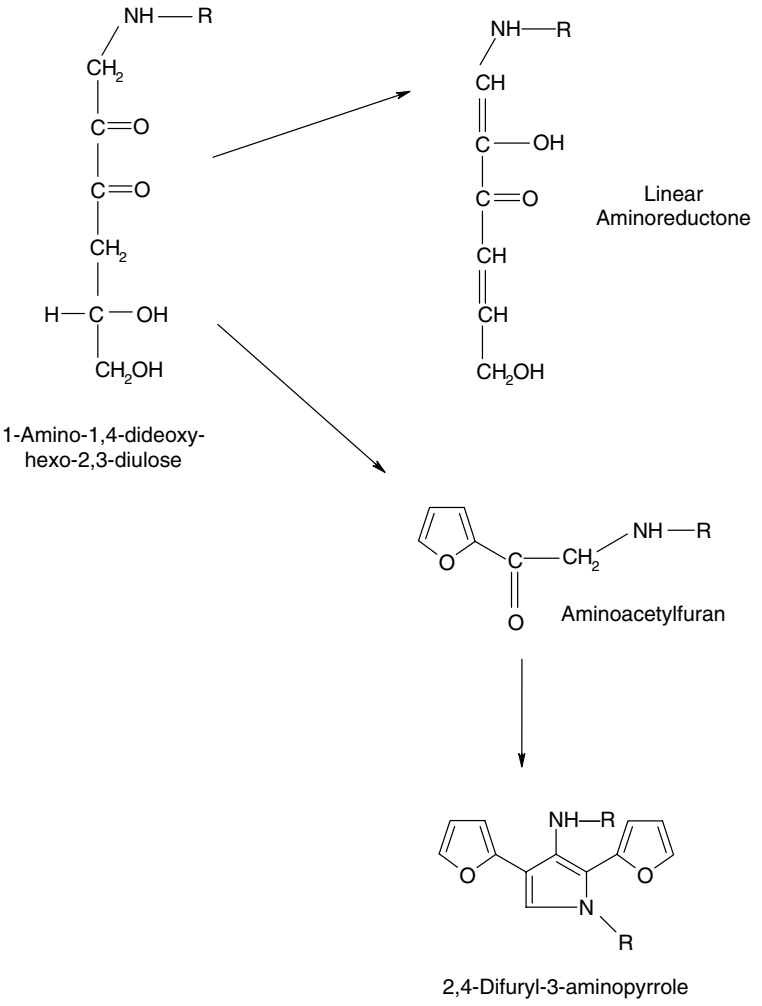

FIGURE 14.20 Degradation of 1-amino-1,4-dideoxy-2,3-diuloses into amino-reductones.

characteristic products in the presence of amino compounds are 1-deoxy derivatives. At higher temperatures, the 1,2- and 2,3-dehydration of ketosamines prevails.

In the presence of oxygen, ketosamines are decomposed into the respective glycos-2-hexosuloses. The reaction is catalyzed by transition heavy metals. The main products of the degradation of 3-deoxy-D-*erythro*-hexos-2-uloses are (*Z*)-3,4-dideoxy-D-*glycero*-hex-3-enos-2-ulose, the respective (*E*)-isomer, and 5-hydroxymethylfuran-2-carbaldehyde (Figure 14.19). Furan and pyrrole derivatives are formed if amine derivatives are present in excess (Figure 14.20). In the presence of amino acids, 1-carboxymethyl-substituted pyrrole-2-carbaldehydes are generated. Protein-bound lysine reacts with 3-deoxy-D-*erythro*-hexos-2-ulose forming pyrrole derivatives. Similarly, analogous compounds are produced by reactions with disaccharides.

Polyhydroxyalkyl-substituted pyrroles are formed by reaction of 3-deoxyhexos-2-uloses with ketosamines. In the presence of ammonia, the analogous pyrazines may form by dimerization of ketosamines. All polyhydroxyalkyl-substituted heterocyclic compounds can convert on heating into the respective alkyl-substituted derivatives.

Other Maillard reaction products, 1-deoxyglyco-2,3-diuloses, are also very reactive compounds, which have not been directly isolated from foods, but their degradation products, furanones, have

been. In the presence of cysteine, different thio derivatives are produced that are responsible for cooked or baked meat aroma.

Aminoreductones are produced by the degradation of 1-dideoxy-2,3-diuloses. They are easily transformed into 1-amino-1,4-dideoxy-hexo-2,3-diuloses, which are isomerized into the respective linear aminoreductones (Figure 14.20). The reaction is reversible, but shifted to the formation of aminoreductone. A parallel transformation product is aminoacetylfuran, which is dimerized into 2,4-difuryl-3-aminopyrrole. Another degradation product of 1-deoxyhexosones is a very reactive compound, diacetylformosin [2,4-dihydroxy-2,5-dimethyl-(2*H*)-furan-3-one], which reacts with primary amines to form substituted pyrrolidones and with secondary amines to form alicyclic aminoreductone. The analogous methylene reductonic acid is generated by reaction of deoxyosones with primary amines, and the analogous reductone acid is produced by interaction of amino acids with pentoses.

The Strecker degradation is a very important reaction of the sugar degradation products with amino acids. The reactive sugar derivatives are α-dicarbonylic compounds, such as glycos-2-ulose or glycos-2,3-diulose, and other simple compounds formed from sugar degradation, for example, glyoxal or methylglyoxal. Strecker degradation results in breakdown of amino acids to aldehydes, ammonia, and carbon dioxide. Each amino acid produces a specific aldehyde with a distinctive aroma (see Chapter 5) that further reacts with amine derivatives with the formation of flavor compounds typical for heated foods.

14.3.2.3 The Effect of Reaction Conditions

Temperature affects both the rate and the mechanism of the Maillard reaction. The activation energy varies between 10 and 160 kJ/mol. The water content has great influence on the activation energy. The browning rate increases with the increasing temperature reaching its maximum in the range of water activities of 0.3–0.7. At low water content, the reaction is slow; it increases with increasing concentration of water, but at much higher water contents, the concentration of reactants is low so that the reaction rate starts to decrease again. The rate of the Maillard reaction increases with increasing pH value, and attains its maximum in slightly alkaline medium. Browning is inhibited by sulfur dioxide and sulfites or sulfur-containing compounds, as sulfites react with aldehyde and keto group or sugars, decreasing their reactivity.

14.3.2.4 Nutritional Significance

The early products of the Maillard reaction involving the lysine residue can be nutritionally utilized in the human organism. Further changes, however, gradually make the amino acid unavailable. Generally lysine is affected mainly in the outer parts of conventionally heated products, exposed to high temperature during baking, grilling, or frying, whereas in the center of the bread loaf or cooked meat, the yield of the reaction products is negligible. However, in various liquid and powdered enteral proteinaceous food formulas produced commercially, the lysine availability may be decreased by up to 25% [11]. Such products are usually made of high-value proteins rich in lysine and the saccharide components may contain substantial amounts of reducing sugars. The same applies also to commercial infant formulas. In some products rich in proteins and reducing sugars, for example, condensed milk, the browning proceeds also at ambient temperature. Although the rate of reaction is much slower, the content of available lysine may drop by a few percent after several months of storage. The loss of nutritionally available lysine can be followed by determining the unchanged amino acid residue, or by assaying some early Maillard reaction products—furosine or N^{ε}-carboxymethyllysine.

It has been suggested that acrylamide may be formed in Maillard-type reactions between glucose and asparagine. In fried and roasted potatoes the concentration of acrylamide increases with the content of fructose and glucose in the raw material. Its content in the fried products can be significantly decreased by washing away the reducing sugars and asparagine from the surface of the cut potato

before frying [12]. Acrylamide interacts readily with the thiol group of cysteine, and, at a lower rate, with amino and hydroxyl groups of different food constituents—providing chemical strategies to curtail acrylamide accumulation.

14.4 REACTIONS DUE TO HEATING IN ALKALINE CONDITIONS

Other types of interactions occur in food systems due to alkaline treatment. Alkalis are applied in food processing for extraction and texturization of proteins from different sources, for example, oilseeds, grains, or bones from meat and poultry carcasses; inactivation of mycotoxins and protein inhibitors; removal of nucleic acids from single cell biomass; pealing of fruits and vegetables or during preparation of tortillas. The first step of the reactions in proteins heated in alkaline conditions is β-elimination in cysteine, serine, phosphoserine, and threonine residues due to attack of the hydroxide ion (see Chapter 5). The nucleophilic additions to the double bond of the dehydroalanine residue, formed as a result of β-elimination, lead to cross-linking of the polypeptide chains and to various unnatural compounds. In hydrolysates of proteins heated at high pH, among other amino acids, a mixture of L-lysino-L-alanine and L-lysino-D-alanine also appear, with probably a small proportion of DL and DD isomers, ornithinoalanine, lanthionine, and methyllanthionine. The products of reaction with ammonia and with phenylethylamine are diaminopropanoic acid and 3-(*N*-phenylethylamino)-alanine, respectively. Recombination of the carboanion with a proton leads to the formation of L and D amino acid enantiomers (see Chapter 5). Prolonged heating at alkaline conditions may decrease the nutritional value of proteins by loss of essential amino acids and racemization; the D-forms are absorbed at a lower rate than the L-forms. The D-enantiomers are not used for protein synthesis. Lysinoalanine chelates Cu^{2+}, Co^{2+}, and Zn^{2+}, thus inactivates metalloenzymes, and induces nephrocytomegaly in rats [13,14]. Also, other products generated due to heating of proteins at alkaline conditions, for example, diaminopropanoic acid and D-serine, are known to induce kidney damage.

A beneficial effect of heating at high pH is the release of nutritionally available niacin from grains. The traditional procedure of Hopi Indians of cooking mature corn grain with alkaline wood ashes hydrolyzed the ester linkages binding niacin to saccharides and turned the nutritionally unavailable polymer into free niacin [15].

14.5 WATER–PROTEIN AND PROTEIN–PROTEIN INTERACTIONS

14.5.1 Water in Proteinaceous Structures

The structural characteristics of proteins in moist food products are affected by their interactions with water via hydrogen bonding and hydrophobic interactions. Thus, protein–water interactions affect the stability of protein molecules and assemblies, for example, the casein micelles, and the functional properties of proteins in food raw materials and products.

Water constitutes about 60–85% of the mass of the tissues of beef, pork, poultry, fish, shellfish, and mollusks while the protein content is only about 12–22%. This large amount of water can be held in the meat against the action of gravity, centrifugal forces, or mechanical compression mainly due to compartmentation in the tissues and because of various interactions with protein molecules. This property of muscle foods, known as water holding capacity or water binding potential, can be characterized either by measuring the expressible liquid from a meat sample by pressing or centrifugation, or by adding water or an aqueous solution to the minced tissue and determining the quantity of water held by the sedimentable material in the centrifuge tube. Any treatment, additive, and biochemical process that favors loosening of the myofibrillar structure by enhancing mutual repulsion between

the fibrils, strands, and sheets increases the water binding potential. Thus, the factors affecting the retention of water in meat systems and thereby the juiciness of the products include the characteristics of the proteins, the pH in the meat, the concentration of postmortem metabolites and added salts, mainly organic and inorganic phosphates, and the changes in proteins due to tenderization, frozen storage, and processing. Several other functional properties, such as gelation, foaming, emulsification, viscosity, and thickening, are also dependent on protein–water interactions and these are discussed in Chapter 5.

14.5.2 Ice Crystal Growth Inhibition by Proteins

Some proteins interact with water molecules in ice crystals. Such interactions enable polar fish to survive in the marine environment during winter. The blood of fishes inhabiting polar oceans, as well as the blood of some insects, contains specific proteins, known as antifreeze proteins (AFP) and antifreeze glycoproteins (AFGP). They are responsible for the noncolligative depression of the freezing point of the blood serum of these animals. This depression is very significantly higher than that predicted from the concentration effect of the polymeric solutes. Thanks to these proteins, the freezing point of the blood serum of some Antarctic fishes is about 1.4°C lower than that of other fishes. Both the AFP and AFGP preferentially bind to the ice lattice and retard the growth of the crystals.

Several types of AFP are known. Various mechanisms for the specific binding of AFP to the ice lattice have been proposed, which postulate involvement of hydrogen bonds, entropic effects, and van der Waals interactions [16]. In winter flounder AFP was studied by Cheng and Merz [17]. The flounder AFP is a single α-helix with a sequence of *Asp-Thr-Ala-Ser-Asp*-Ala_6-Leu-*Thr-*Ala_2*-Asn*-Ala-Lys-Ala_3-Glu-Leu-*Thr-*Ala_2*-Asn*-Ala_7*-Thr-Ala-Arg*; there are four ice-binding regions (here marked in bold characters). As has been shown, the grouping of these regions along the polypeptide chain is compatible with the ice surface topology. Therefore, the residues in all ice-binding regions simultaneously form five to six hydrogen bonds each with the water molecules in the crystals. The proper sequence of the amino acid residues is also necessary for the van der Waals interactions and the hydrophobic effect to participate in the binding of the protein. Antifreeze proteins have potential uses in frozen foods where they can inhibit ice crystal growth and retard the rate of ice recrystallization.

14.5.3 Protein–Protein Interactions

Foods contain a large variety of proteins. This applies to each individual protein source. For instance, in milk there are caseins, α-lactalbumin, β-lactoglobulin, serum albumin, immunoglobulins, and proteose-peptones; meat contains myofibrillar proteins, collagens, and the sarcoplasmic proteins—mainly enzymes; cereal grains contain a mixture of albumins, globulins, prolamins, and glutelins. Proteins differ in size, shape, and amino acid composition and sequence. As a result, they differ in their functional behavior in food systems. Interactions of various reactive groups, such as thiol groups, and hydrophobic patches exposed on the surface of protein molecules lead to the formation of covalent as well as noncovalent intermolecular links, which result in protein aggregation and/or polymerization. Such molecular changes alter the functional properties of proteins, such as hydration, solubility, viscosity of solutions, film formation, gelling, and adsorption on the interface between aqueous and lipid phases (see Chapter 5).

14.6 PROTEIN–LIPID INTERACTIONS

In oil-in-water emulsion-type products, such as milk, cream, ice cream, cheese, dressings, mayonnaise, and various comminuted meat products, the structure-forming role is played by lipid droplets distributed in an aqueous continuous phase. These droplets are stabilized by interactions

with proteins, lecithins, or synthetic surfactants. During storage of chilled milk, interaction of the lipoprotein membranes of fat globules with a serum protein leads to clustering/flocculation and creaming of fat globules. In contrast, clustering of fat globules around air bubbles contributes to the desirable textural characteristics of ice cream.

In water-in-oil emulsion-type foods, the structure-forming role is played by water droplets that are stabilized by interactions with solid fat particles, monoacylglycerols and phospholipids, or proteins that accumulate at the water–lipid interface. Depending on their size and distribution, they affect the rheological and sensory properties, as well as microbial stability of such products.

Wheat flour contains a number of low molecular weight proteins that are rich in cysteine and basic amino acid residues. Thionins, ligoline, lipid transfer proteins, and puroindolines belong to this class of proteins. In the native state, they can spontaneously bind lipids or lipid aggregates. These lipid-binding proteins facilitate the spreading of lipids as a monolayer at the air–water interface. Puroindolines are known to be very efficient foam stabilizers. Interactions of puroindoline-*a* with lysophosphatidylcholine have a synergistic effect on the foam stability of the system [6].

14.7 POLYSACCHARIDE INTERACTIONS IN FOOD SYSTEMS

Interactions of polysaccharides with each other and with ions, proteins, and lipids affect various functional properties in food systems; these include water holding capacity, gelling, film-forming, viscosity, and other rheological behavior of fluids, inhibition of crystal growth, as well as formation and stability of foams and emulsions. These properties contribute to the sensory properties of meat and fish products, milk gels, custards, creams, ice creams, salad dressings, cakes, bakery fillings, jams, marmalades, jellies, fruit drinks, and instant beverages. Modified starches are widely used as emulsion stabilizers and thickening agents. Polysaccharides also serve as entrapping or complexing agents for gases, liquids, and solids in foods. The interactions involved comprise surface sorption, and inclusion of the guest molecules within the amylose helix and short helices of amylopectin, as well as in the capillaries between starch granules [18].

The microstructure of starch granules consists of assemblies of six double helices of amylose in a hexagonal unit with a channel in the center harboring about 36 water molecules. When native granular starch is soaked in water, it swells due to absorption of up to 30% of moisture by weight, mainly in the amorphous region of the polymer. The swelling is reversible; the absorbed water can be removed by drying at ambient temperature. On heating, the swollen granules lose their double helices and consequently the crystalline structure and turn irreversibly into gelatinized starch. The gelatinization temperature is in the range of 50–70°C and is different for starches isolated from various botanical sources (Chapter 3). It increases in the presence of salt and some low molecular mass hydrophilic components, which is the result of competition of these small constituents with starch for available water molecules. Further heating and shearing in excess of water leads to increased swelling of the granules and formation of starch pastes of high viscosity. On cooling, the starch paste develops a network, leading to further increase in viscosity, and the highly hydrated mass turns into a gel.

During prolonged storage of the paste or gel, the double helical crystalline structure gradually develops again and the polysaccharide chains aggregate, whereby intermolecular hydrogen bonds are involved. This process is known as retrogradation. Such retrograded starch has lower water holding capacity. The rate of retrogradation depends on the type of starch, that is, the ratio of amylose and amylopectin content, the lipid content, and storage temperature [9]. Amylose gels retrograde readily within hours, whereas amylopectin gels take days or weeks. Retrogradation is manifested in bread by staling (see Section 14.12.4), while in gels it leads to the formation of dendrites.

The hydration of neutral polymer networks depends on the intensity of interaction of the material with the solvent. The immobilization of water molecules is to a large extent due to hydrogen bonding

with the hydrophilic groups of the polysaccharide or hydrocolloid. On the other hand, in the case of gels of polyelectrolytes, such as pectins, the swelling is affected by accumulation of counter ions within the polymer network, whereby different effects may be produced. The counter ions, in accordance with the requirement for electrical neutrality, accumulate within the gel in concentration higher than that in the external medium. This leads to an osmotic pressure difference between the gel and the medium and results in increased swelling. The osmotic pressure effect depends on the charge on the polymer and on the ionic strength. On the other hand, cross-linking of pectin chains via Ca^{2+}-mediated electrostatic bonds may counteract network expansion and decrease swelling (i.e., Ca^{2+} firming) [19].

In plant cell walls, the cellulose fibers are cemented to one another by hemicelluloses and pectic substances. Cellulose remains insoluble during cooking, while hemicelluloses become partially dissolved. The solubility of hemicelluloses can be promoted by increasing the pH of the cooking medium. Therefore, vegetables, especially pulses, that tend to remain firm when boiled in acidic or neutral conditions, become soft in alkaline aqueous solutions. Thus $NaHCO_3$ is sometimes added to water used for boiling pulses. The significance of the effect of pectic substances depends on the ripeness of the vegetable. During ripening, the insoluble protopectins are enzymatically converted into soluble pectins. In alkaline medium the free carboxylic groups of pectins form soluble salts, which contribute to the softening of cooked vegetables.

The interactions of water with various plant hydrocolloids (gums) generally do not lead to gelling. Instead, even at low concentrations, a very viscous, pseudoplastic solution is formed due to the large molecular size of the polymers. The rheological behavior of solutions of neutral gums is pH-stable, while the viscosity of solutions of polymers with ionizable groups depends significantly on the pH of the system [20].

Polyanionic hydrocolloids bind electrostatically the cationic counter ions. Depending on the type of the anionic group of the polysaccharide, that is, $-COO^-$, $-PO_3^{2-}$, or $-OSO_3^-$ and the properties of the cations, the counter ions are either bound along with their hydration shells or after displacement of the hydration shell. Thus, the binding affinity of various cations to different anionic hydrocolloids, for example, carboxymethylcellulose (CMC) or carrageenan, must be taken into consideration in formulating gum additives in food systems.

Polymer–polymer interactions in mixed polysaccharides solutions often lead to increased viscosity of the solutions and, in some cases, gelling. The extent of synergism depends on the chemical composition and structure of the respective hydrocolloids. The gelling of agarose, carrageenan, and furcellaran can be enhanced by addition of other, nongelling polysaccharides. For example, the addition of locust bean gum and other galacto- and glucomannans to κ-carrageenan increases the gelling ability of the system. Several hypotheses have been proposed to explain this phenomenon: these include intermolecular hydrogen bonding between the two interacting polysaccharides, self-aggregation of the galactomannan chains in the presence of the κ-carrageenan network, and excluded volume effect arising from incompatibility of mixing of the polymers. Addition of gluco- or galactomannans to xanthan gum has a synergistic effect on its ability to form thermo-reversible gels even at 0.5% total polysaccharide concentration. This effect is probably due to specific interactions between the xanthan macromolecules and unsubstituted (smooth) sequences of the other polymer. The properties of alginates and of high-methoxyl pectin gels can be controlled by exploiting the synergistic action of these polymers on each other. For instance, whereas the alginates alone can form firm gels in the presence of multivalent cations but precipitate at low pH, the pectins form gels in absence of cations, at low pH, and in the presence of about 40% sucrose. On the other hand, a 1:1 mixture of alginate and pectin at 0.6% forms a gel at low pH and at high-soluble solids content. This synergism is probably due to interchain associations of these polymers; the polyguluronic acid (alginate) and the methyl esterified polygalacturonic acid (pectin) can pack together in parallel twofold crystalline arrays [21]. Interactions of starch with various gums may also change the viscosity, gel set rate, and rheological properties of the gels.

14.8 POLYSACCHARIDE–LIPID INTERACTIONS

Polysaccharide–lipid interactions in aqueous systems are due to hydrophobic effects. A typical example is binding of a monoacylglycerol molecule in the interior cavity of the helical structures of amylose. An amylose–lipid complex is formed that has a structure similar to that of the well-known amylose–iodine complex. In dough the complexing of lipids by amylose reduces the swelling of starch granules and increases the gelatinization temperature [8]. Heating applied during baking increases the extent of binding of lipids by amylose. The amylose–lipid complexes have the ability to form gels. The rheological properties of these gels depend on the concentration and type of lipids and on the crystalline form of the complexes. Addition of lipids, especially phospholipids, retards retrogradation of starch gels and softens the bread-crumb. The mechanism involves interaction of lipid micelles with the hydrophilic segments of starch. Such interactions also promote gelling of many starch systems [22].

Hydrocolloid–lipid interactions are the basis of the excellent emulsifying properties of gum Arabic. Although this polysaccharide has a high molecular mass, 260–160 kDa, its solutions have very low viscosity. Both these properties are the result of the unique composition of gum Arabic. It is a mixture of polysaccharides composed of L-arabinose, L-rhamnose, and D-glucuronic acid, which form three components—a high molecular mass arabinogalactan–protein complex (AGP), a glycoprotein, and an arabinogalactan. In the AGP several compact hydrophilic polysaccharide structures are covalently linked to a large polypeptide chain that is probably located at the periphery of the complex. This structure, with the hydrophobic amino acid residues directed toward the lipid phase, makes the adsorption of the gum at the oil–water interface possible. Heat denaturation of the proteinaceous component results in a loss of the emulsifying capacity of the gum.

Polysaccharide–lipid interactions can be utilized in manufacturing low-calorie emulsion-type food products. An aqueous dispersion of 2% microcrystalline cellulose is similar in respect to viscosity as a 60% oil-in-water emulsion, apparently due to the formation of an internal three-dimensional network. An emulsion of 20% soybean oil in an aqueous solution containing 1–1.5% colloidal microcrystalline cellulose and 0.5% polyoxyethylene sorbitan monostearate emulsifier can simulate the properties of a 65% pure oil emulsion in terms of viscosity, yield value, flow behavior, and stability [21].

14.9 POLYSACCHARIDE–PROTEIN INTERACTIONS

Some hydrocolloids interact with proteins. In dairy products, such interactions may lead to destabilization of the proteins and/or prevent undesirable precipitation by Ca^{2+}. The neutral polysaccharides, such as locust bean gum and guar gum, as well as most polyanionic polymers, including the slightly acidic gum Arabic, CMC, pectin, hyaluronic acid, alginates, agarose, heparin, chondroitin sulfates, cellulose sulfate, and fucoidan, are not effective against precipitation of casein micelles and α_{S1}-casein by Ca^{2+} at pH 6.8. On the other hand, carrageenans, especially the κ-carrageenan, form stable complexes with α_{S1}-casein. However, the nature of this interaction is not clearly understood [21].

Biopolymers do not mix well with each other in concentrated solutions. Because of this thermodynamic incompatibility of mixing, a concentrated aqueous solution mixture of biopolymers separates into two aqueous phases, which is generally referred to as water-in-water emulsion [23]. The concentration threshold for phase separation depends on the types of biopolymers in the mixture. The mixtures of globular proteins phase separate only at above a minimum concentration of 12%, whereas mixtures of protein and polysaccharide separate at a much lower concentration of about 4% (Figure 14.21). When two biopolymer solutions are mixed, the resulting mixture remains as a homogeneous isotropic solution if the total concentration of the biopolymers is below the threshold concentration. However, above the threshold, the solution separates into two phases: initially, depending on the phase volume, one aqueous phase enriched in one of the biopolymers

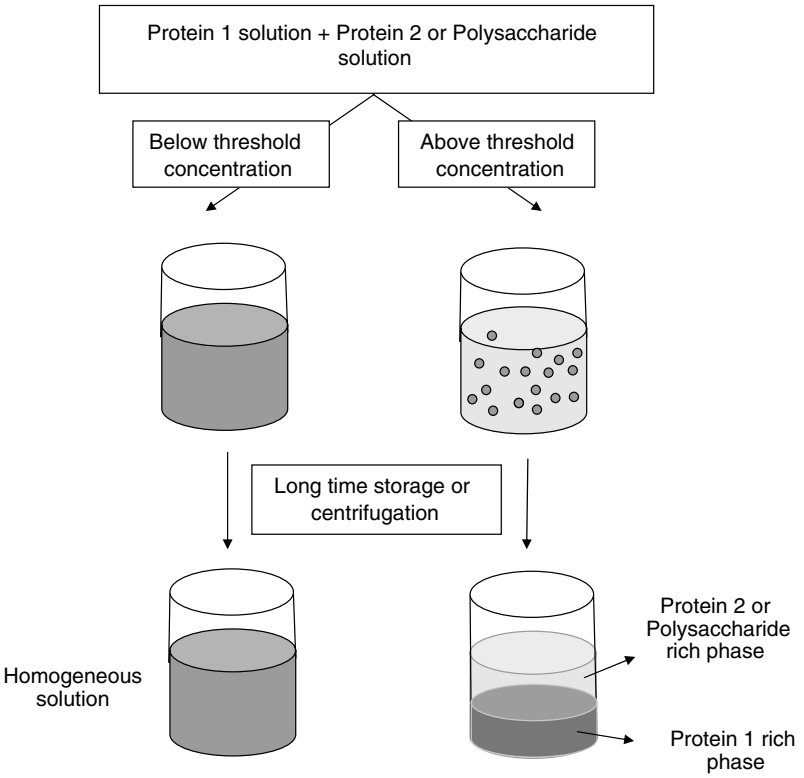

FIGURE 14.21 Separation of concentrated aqueous solution mixture of biopolymers into two aqueous phases. (Courtesy of Tolstoguzov, V. (2000). *Nahrung*, 44: 299–308.)

becomes dispersed in an aqueous continuous phase enriched in the other. Since both these phases are aqueous, visual separation is not apparent. When this system is allowed to stand or is centrifuged, the water-in-water emulsion separates into two regions, with the top of the aqueous solution enriched in one of the biopolymers and the bottom of the solution enriched in the other.

A typical phase diagram of protein–protein and protein–polysaccharide solution mixtures at equilibrium is shown in Figure 14.22. The binodal curve represents the equilibrium concentrations of the biopolymers in the phase-separated regions. For example, suppose that a pure solution of protein 1 at concentration B is mixed with a solution of protein 2 (or a polysaccharide) at concentration A such that the composition of the mixed solution is represented by M. Upon standing or centrifugation, the solution mixture with composition M breaks into two phases, with the top phase having a composition represented by D and the bottom phase with composition represented by E. The line DE is known as the tie line. The volume ratio of these two phases is given by DM/ME. The binodal phase diagram for a biopolymers mixture is obtained by mixing the two biopolymer solutions at various concentrations and determining the equilibrium composition of the two phases. A set of tie lines can be obtained for each phase diagram by connecting the equilibrium composition of the two phases (i.e., Ds and Es). The utility of such a phase diagram is that it provides information on the concentration ranges at which a mixture of biopolymers is thermodynamically stable or unstable. For instance, at all total concentration of biopolymers below the binodal curve, the mixture is thermodynamically stable and no phase separation would occur, whereas at all total concentration above the binodal curve the system is thermodynamically unstable and would spontaneously separate into two phases. There is a single point on the curve at which the total concentration of the biopolymers is the minimum. This point represents the threshold concentration (T) for a given biopolymer mixture above which phase separation would occur. This threshold concentration is typically about 4% for a globular protein +

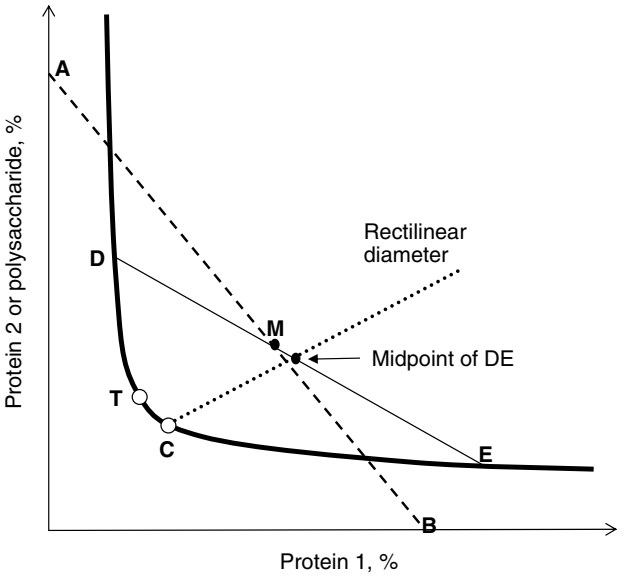

FIGURE 14.22 A typical phase diagram for a binary mixture of two biopolymers in aqueous solutions. DE, tie line; T, threshold composition; C, critical point. (Courtesy of Tolstoguzov, V. (2000). *Nahrung*, 44: 299–308.)

polysaccharide mixture and above 12% for globular protein + globular protein mixtures. In the case of gelatin + polysaccharide mixtures, it is in the range of 2–4% depending on the molecular weight distribution of gelatin. Another useful parameter that can be derived from the phase diagram is the critical point (C). This represents the composition of the mixture at which the two phases have equal volume and same composition. This point can be determined from the intersection of the line passing through the midpoints of the tie lines and the binodal curve. The line connecting the midpoints of the tie lines (for clarity, only one tie line is shown in Figure 14.22) is referred to as the rectilinear diameter.

Thermodynamic incompatibility of mixing and phase separation in biopolymer mixtures is mainly due to the excluded volume effect (Figure 14.23). The size the biopolymers A and B represent their radius of gyration. These particles can approach each other up to a minimum distance equivalent to the sum of their radii. Since molecules are not interpenetrable and the segments of two biopolymers cannot simultaneously occupy the same space, they exclude each other from their respective space, shown as shaded area in Figure 14.23. This space includes the hydration layer around each molecule; thus, the excluded volume around each molecule is accessible only for the solvent molecules but not for the other polymer. When the concentration of A is increased, the space available for B in the solution decreases because of an increase in the total excluded volume. Hence molecules of B are forced to segregate and separate into a phase, carrying with them a portion of the solvent. Random-coil biopolymers, such as gelatin, have much larger excluded volume than a globular protein of similar mass. Therefore, they induce phase separation at a much lower concentration than globular proteins. This also is the case with polysaccharides.

Phase separation in biopolymer mixtures can also occur via a process known as "depletion flocculation." Consider a random-coil biopolymer, gelatin or a polysaccharide, in a solution of a globular protein (Figure 14.24). When the space available between two globular protein molecules is less than the entropically favorable expanded size of the random-coil polymer, it excludes itself from that space. This creates a local concentration gradient, which in turn develops an osmotic pressure gradient; as a result, the globular protein molecules are forced into one region and the random-coil polymer molecules into another region of the solution, causing phase separation.

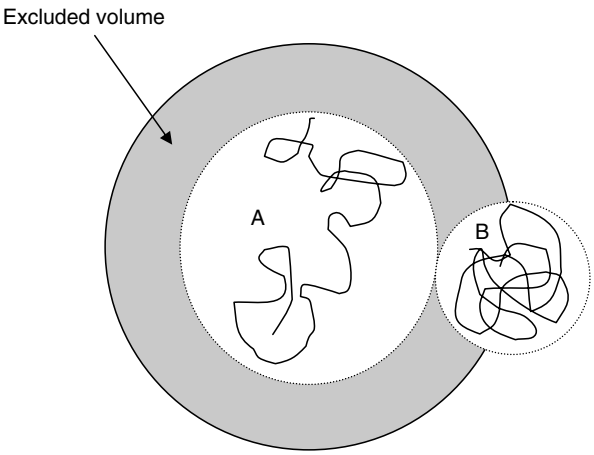

FIGURE 14.23 Schematic illustration of volume exclusion in biopolymer mixtures. (Courtesy of Tolstoguzov, V. (2000). *Nahrung*, 44: 299–308.)

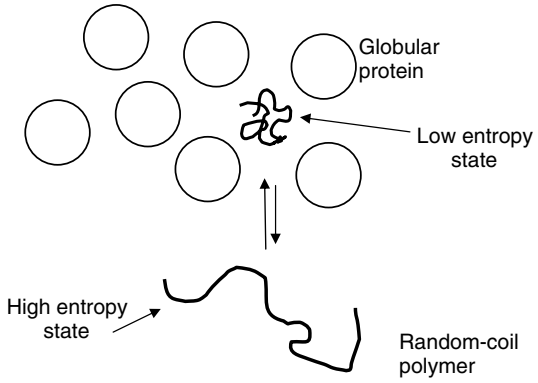

FIGURE 14.24 Schematic representation of the depletion flocculation mechanism for phase separation in biopolymer mixtures. (Courtesy of Tolstoguzov, V. (2000). *Nahrung*, 44: 299–308.)

Since most foods contain mixtures of proteins and polysaccharides at concentrations greater than the threshold concentration, phase separation is the rule rather than exception. The extent of such phase separations during formulation of a food material can impact on the final sensory properties of the product after processing. An example of how such phase separation can affect the textural properties of gels is depicted in Figure 14.25. Phase separation results in heterogeneous distribution of biopolymers in food formulations, which upon processing creates heterogeneity in the texture of the gel, which may or may not be desirable depending on the product.

14.10 THE EFFECT OF INTERACTIONS ON THE COLOR OF FOODS

14.10.1 Changes in Hemoproteins

The color of meat of slaughter animals, fish, mollusks, and crustaceans is affected by the content and the chemical state of hemoproteins, mostly myoglobin (MbFe(II)), and to a lesser degree hemoglobin,

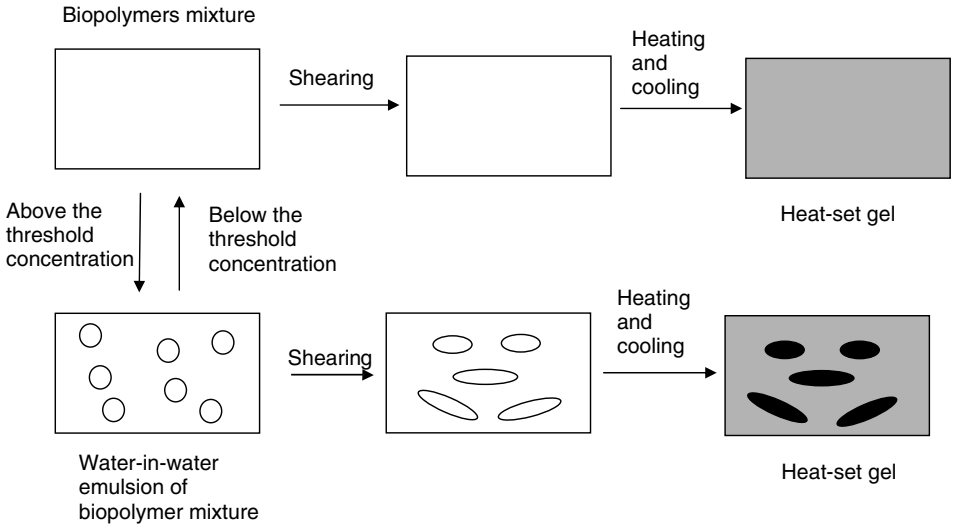

FIGURE 14.25 An example of how such phase separation can affect the textural properties of gels. (Courtesy of Tolstoguzov, V. *Nahrung*, (2000). 44: 299–308.)

cytochromes, and hemocyanin. The chemistry of color changes in hemoproteins is described in detail in Chapter 16.

14.10.2 Interactions of Carotenoids

Carotenoid pigments are responsible for the color of many vegetables and fruits. These are often mixtures of up to several dozens of compounds (see Chapter 7 for the sources of various carotenoids). Carotenoids easily undergo oxidation in the presence of air and light but are fairly stable at cooking temperatures. Oxidation of carotenoids during storage and processing of foods leads to the loss of the characteristic color of many food products and food commodities. A typical example is the appearance of brown discoloration on stored red peppers due to oxidation of capsanthin. A further result of oxidation of carotenoids is the development of aroma compounds [24].

The skin of many fish and the shell of marine crustaceans are distinct because of their vivid rainbow colors. They are yellow, orange, red, purple, blue, silver, or green. The major pigments are various carotenoids, such as astaxanthin and canthaxanthin, which exist as noncovalent complexes with proteins, glycoproteins, phosphorylated glycoproteins, glycolipoproteins, and lipoproteins [25]. Dissociation of the carotenoprotein complexes during storage of fish, especially in direct bright light, leads to fading of the colors.

The major carotenoid component in the carapace of lobster is the red astaxanthin. In the live animal, it is present in the form of a water-soluble carotenoprotein crustacyanin, which is blue. Thus, the fresh lobster has a blue or blue-gray color. However, during boiling the crustacean in water, the protein complex is denatured and free astaxanthin is released, yielding the bright red color.

14.10.3 Interactions of Anthocyanins

Anthocyanins, glycosides of polyhydroxy, polymethoxy derivatives of the flavylium cation, form a large group of dominant pigments present in fruits and flowers (see Chapter 9). Their red, violet, or blue color depends on their structure and on the pH of the aqueous media. Hydrophobic interactions and hydrogen bonding between different anthocyanins and with some other phenolic acids and with alkaloids present in various plants affect the intensity of color and change the light absorption

wavelength. In red wines, the changes in color during maturation are caused by condensation reactions between anthocyanins and other phenol compounds, as well as due to the formation of other anthocyanin pigments in reactions with pyruvic acid. The various derivatives may be blue, yellow, orange, orange-red, red, and brown [26].

The stability of anthocyanins in food products is rather poor. They are susceptible to enzyme-catalyzed degradation, light, heat, oxygen, acidity, and oxidative degradation of ascorbic acid. During prolonged storage of pasteurized fruit juices, the color may gradually fade or turn brown due to polymerization reactions. In the presence of Al, Fe, and Sn, purplish-blue or slate-gray pigments may be formed in processed fruits [24].

14.10.4 Nonenzymatic Browning and Black Spot Formation

The compounds formed in the early stages of Maillard reactions are essentially colorless or slightly yellowish. They are called premelanoidins as they are precursors of brown-colored melanoidins. The colorless low-molecular weight products (<1 kDa) are produced mainly by reactions with free amino acids. Brown-colored macromolecular polymeric Maillard products—melanoidins—are generated predominantly by polymerization of intermediary products and/or by reactions with the reactive groups of amino acid residues in proteins. The latter mechanism is more frequent in foods. Reactions of oxidized lipids with proteins result in the formation of colored compounds similar to those produced in Maillard or enzymic browning reactions. Lipid peroxy radicals, lipid hydroperoxides, or their decomposition products interact with free amino acids forming yellow to brown products. The browning rate is particularly high in reactions with cysteine, methionine, and tryptophan. Similar reactions can also occur between oxidizing lipids and proteins. Since unsaturated lipids are more prone to oxidation than saturated ones, the browning reaction is more intense in tissues rich in fats containing polyenoic fatty acids, such as fish muscle. The browning discoloration is objectionable especially in white poultry or fish muscle, where brown spots are produced even under refrigerated or frozen storage.

The brown or even black discoloration can also result from enzymatic reactions. The color of the shells and surface layers of crustaceans, for example, lobsters, shrimps, and crabs, deteriorates after catch by melanosis, manifested as very dark or black spots. The enzymatic darkening is initiated by endogenous polyphenol oxidase (PPO) and proceeds further via nonenzymatic polymerization. In the enzymatic step, the monophenol oxidase catalyses the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) and the oxidation of DOPA is catalyzed by diphenol oxidase. The DOPA undergoes polymerization to form high molecular weight black pigments. DOPA can also react with cysteine, tyrosine, and lysine residues in proteins and thereby involve proteins in the polymerization reaction. Extensive blackening is a serious quality defect in crustaceans. To prevent the formation of black spots, various sulfiting agents are used to reduce the quinone to the colorless DOPA. Similar reactions also occur in bananas, figs, peaches, apples, potatoes, nuts, and cereal products. Since they depreciate significantly the sensory quality of many foods, they are responsible for great economic losses in the food industry.

14.10.5 Discoloration Owing to Binding of Metal Ions

Most foods contain traces of transition metals, especially Fe^{2+} or Fe^{3+} and Cu^{2+}. During food storage or heating, dissolved metals are distributed between the lipid and aqueous phases. Those ions, which have been dissolved in the lipid phase, are mainly bound to fatty acids as nondissociated salts or to phospholipids. This makes the color of the lipid phase orange brown. The ions in the aqueous phase may react with phenolic substances, forming colored complexes. For instance, complexes of Fe^{2+} with pyrocatechol or pyrogallol derivatives are blue or violet, while analogous complexes with Fe^{3+} ions are orange or brown.

14.11 INTERACTIONS AFFECTING FOOD FLAVOR

14.11.1 Interactions of Flavor Compounds with the Main Food Components

The perceived flavor of a food is related predominantly to the concentration of volatile flavor compounds in the vapor phase above the product. The flavor is due to vapors ascending from the oral cavity to the nasal cavity during chewing a morsel of food. The vapor phase concentration depends at any given temperature on the concentration of the volatile compounds in the food material, on their volatility, mostly influenced by water vapor from saliva, and on their affinity to other components of the food.

Flavor compounds in foods are entrapped by proteins, lipids, or polysaccharides. The binding may be due to hydrophobic interactions, as well as hydrogen, ionic, or covalent bonds, depending on the structure of the volatiles and of the entrapping compounds. Entrapment decreases the volatility of the molecules, thus reducing the rate of loss of flavor during storage of the products. The stability of the system depends on the pH and temperature of food. Many aroma compounds are hydrophobic or at least have significant hydrophobic fragments. Thus in a food product they accumulate selectively in the lipid phase.

Hydrocolloids are known to decrease the perceived intensity of various flavors in food systems. Depending on the properties of the flavor compounds and of the hydrocolloids the suppression of the aroma intensity may be due to binding of the volatile compounds or decrease in diffusion rate due to increased viscosity or entanglement within the polymer network. In bread, many flavor compounds are entrapped within the amylose helices. Heating of a bread slice liberates the captured compounds, thus increasing the aroma intensity.

One of the ways to inhibit undesirable reactions of flavor additives with food components during storage, and to protect added compounds against evaporation and chemical degradation during processing, is encapsulation by forming flavor–cyclodextrin molecular inclusion complexes. In these microcapsules, the hydrophobic fragments or the whole flavor compound molecules are entrapped within the hydrophobic cavity of the cyclodextrin while the exterior hydrophilic portion of the cyclodextrin provides water solubility and dispersibility. The formation of inclusion complexes is controlled by the concentration of the guest molecules and the dimensional compatibility of the flavor compounds with the cyclodextrin cavity. Complexation significantly improves the stability of various flavors, especially in products processed at high temperature, for example, hard candy. However, it may also cause poor release of some flavor compounds from the cyclodextrin trap during consumption of the products [27].

Proteins are known to bind various flavor compounds by hydrophobic interactions. Many reports on the binding of alcohols, aldehydes, ketones, and other volatile compounds by various proteins, especially soybean and milk proteins have been published [28].

The difference in the volatility of various flavor compounds present in food is one of the reasons for aroma changes of many commodities during storage.

14.11.2 Hydrolytic Reactions

Changes that occur in bovine meat during aging bring about not only the desirable tenderization, but also increase the contents of free amino acids. The accumulation of amino acids may enhance the flavor formation in the roasted meat. The increase in the concentration of free amino acids in tenderized meats is caused mainly by endogenous proteolytic enzymes from lysosomes. Electrical stimulation of beef carcasses may enhance the release of these enzymes, and thus increase the accumulation of free amino acids. Furthermore, amino acids may be liberated due to the activity of transaminase. Enzymatic transfer of the α-amino group of alanine to 2-ketoglutaric acid may be responsible for the increase in the concentration of glutamic acid in aged beef [29].

14.11.3 Oxidative Reactions

Nitrate used as the curing agent in meats also acts as a potent antioxidant in cooked meat systems. It has been proposed that its antioxidant activity might be based on several mechanisms, including inhibition of the Fe-catalyzed oxidation by metal–ion complexation. It has been shown that nitrite reacts readily with malondialdehyde, the secondary lipid oxidation product, and produces high molecular weight compounds [30]. This reaction limits the usefulness of the 2-thiobarbituric acid test for measuring the oxidative deterioration of lipids in cured meats.

The role of oxidized lipids in the development of rancidity is well known. The rancid compounds, especially carbonyl products, react with amino acids and proteins with formation of very objectionable off-flavors. In the presence of polyenoic fatty acids, 2,4-alkadienals and conjugated alkatrienals are formed, which react with amino acids and produce typical fishy off-flavors.

Terpenes are present in the majority of foods of plant origin. They are easily oxidized upon storage and heating, with the formation of several aroma notes. Oxidized terpenes and products of decomposition of the intermediary hydroperoxides, including radicals, react with amino acids and proteins forming numerous off-flavor compounds.

14.12 INTERACTIONS THAT AFFECT THE TEXTURE AND RHEOLOGICAL PROPERTIES OF FOODS

14.12.1 Freeze Denaturation of Proteins

In frozen stored fish, predominantly of the *Gadidae* family, cross-linking of myofibrillar proteins leads to so-called freezing denaturation. This results in undesirable toughness of the meat and loss of functional properties, mainly water holding, fat emulsifying, and gel forming capacity, as well as in decreased ATPase activity, which is indicative of the undesirable protein changes. The extent of these changes increases with time and temperature of frozen storage. The cross-linking of proteins is caused by the reaction of formaldehyde, generated particularly in the meat of fish belonging to the *Gadidae* family by the endogenous trimethylamine *N*-oxide demethylase, with reactive groups in proteins. Oxidizing lipids may also contribute to protein cross-linking via free radical polymerization and via protein amine condensation with dialdehydes produced as secondary lipid oxidation products. Natural antioxidants (except ascorbic acid), which are often crude mixtures of plant extracts, are effective in inhibiting the undesirable changes in frozen fatty fish. The freezing denaturation is especially severe in minced fish, since rupturing of the muscle structures favors interactions of the enzymes with their substrates and increases lipid oxidation, especially if the mince contains dark meat rich in heme compounds.

These undesirable interactions can be minimized by extracting the water-soluble proteins and nonprotein nitrogenous compounds, including trimethylamine *N*-oxide, many pigments, prooxidative compounds and ions, as well as by removing most of the lipids. The remaining myofibrillar protein concentrate, known as surimi, has much better functional properties than the original material and is more resistant to freeze denaturation. However, during surimi manufacture, about 30% of crude protein is lost from the fish meat.

Further inhibition of the cross-linking reactions can be achieved by adding various compounds that decrease the freezing temperature, increase the viscosity of the tissue fluids, or interact selectively with amino acid residues of proteins. The most common cryoprotectors in surimi manufacture are sucrose, sorbitol, mannitol, alginates, polyphosphates, citrates, ascorbate, and sodium chloride in different, proprietary mixtures. Several amino acids, hydroxy carboxylic acids, branched oligosaccharides, and adenosine nucleotides have been shown to be effective in model systems and in surimi. Some data indicate that among the amino acids, those with high negative net charge are most effective in preventing freezing denaturation of fish myofibrillar proteins. Generally the low molecular weight compounds suitable as cryoprotective substances contain more than one reactive

group, suitably spaced in the molecule. Cryostabilization of proteins in surimi has been reviewed by Matsumoto and Noguchi [31].

14.12.2 Cross-Linking in Gels

Food gels may be made from proteins and polysaccharides via noncovalent, and sometimes covalent interactions, between various functional groups on the polymer chains. Interactions with endogenous or added low molecular compounds may also be involved. The polymers may interact when they are in solutions, dispersions, micelles, or in comminuted tissue structures. Cross-linking in food gels is covered in Chapter 5 on proteins.

Thermal gelation of proteins is crucial for the desirable texture of cooked sausages and gelled fishery products known as kamaboko. In producing kamaboko, which is made of surimi, a two-step heating procedure is often used, that is, setting at about 40°C to allow for cross-linking catalyzed by endogenous transglutaminase, followed by final heating to the required temperature. The gel forming ability of the myofibrillar proteins of fish belonging to various species depends significantly on the endogenous proteinase activity. Owing to excessive proteolysis in the meat of fish of different species, for example, Pacific whiting, Alaska Pollack, New Zealand hoki, or Atlantic croaker, undesirable softening of gels made of surimi occurs. Adding proteinase inhibitors from various sources, for example, beef plasma protein, bovine serum albumin, egg white solids, or potato protein extracts, can arrest these adverse changes of the gels.

14.12.3 Formation of Biodegradable Films

Biodegradable films may be used in food packaging if they have suitable barrier and mechanical properties and because they do not pollute the environment as much as some other materials. In food processing they may be applied as edible carriers of ingredients and antimicrobial agents, and as enzyme supports. They can be made of various polysaccharides, proteins, or protein–polysaccharide and protein–lipid mixtures. The properties of these films depend on the characteristics of the components and on the interactions between the polymers and other compounds.

The various steps involved in the preparation of edible proteinaceous films include initial denaturation of the molecules by heating, shearing, or adsorbing thin layers of material at the air–water interface. In the dope containing the denatured polymers at appropriate concentration, cross-linking is induced between the polypeptide chains by evaporating the solvent or by shearing in a spinneret and immersing in a coagulating bath.

The bonds participating initially in the formation of a cohesive and viscoelastic film are the same as those responsible also for gelling of proteins and polysaccharides. Proper selection of the polymers used for production of multicomponent films and the parameters of the manufacturing process may favor specific interactions, as is shown in the following few examples.

Chitosan in slightly acid environment can form, owing to its positive charge, electrostatic cross-links with alginates, pectins, and other acidic polysaccharides. The tensile strength and barrier properties of films made of mixtures of proteins depend on the number of various reactive groups in the components and on the pH of the dope. In proteinaceous dopes heating at alkaline pH may increase the number of –SH groups, which during drying of the film may form new disulfide cross-links. This treatment results in increased tensile strength, lower solubility in water, and reduced permeability of the films for oxygen, aroma compounds, and oils. Ionizing radiation used in appropriate doses may induce cross-linking in protein films due to the reactions of the generated hydroxyl radicals with tyrosine residues. Additional chemical or enzymatic cross-linking may be induced in order to produce material of desirable plasticity, sufficient tensile strength in various conditions of humidity and temperature, low solubility in hot water, controlled barrier properties, and defined biodegradability. The properties of materials made of anionic polymers may be modified by adding bivalent cations. For chemical modification of proteinaceous films, predominantly cross-linking reagents

are used, which react with the amino groups of amino acid residues, mainly various aldehydes, or *N*-[3-(dimethylamine)propyl]-*N′*-ethylcarbodiimide (EDC). However, in selecting chemical cross-linking reagents toxicological aspects must also be considered. Transglutaminase-catalyzed cross-linking may be regarded as a useful enzymatic method of choice for modifying proteinaceous films. By using the enzyme treatment or EDC, the solubility of fish gelatin or gelatin–chitosan films in boiling water, even at acid pH, can be effectively reduced and the tensile strength increased [32].

14.12.4 Interactions in the Dough and Baked Loaf

The quality of many products of the baking industry depends to a large extent on the formation in the dough of various protein cross-links and protein–lipid–polysaccharide interactions that are necessary for the desired porous, aerated texture of these commodities. The cross-links are formed during mixing of the flour with water and the added salt, as well as at further stages of bread-making. The dough can be regarded as a viscoelastic, hydrated matrix of gluten proteins with embedded starch granules, cell wall debris of various sizes, as well as neutral and polar lipids, air, and fermentation gases.

Mixing with water leads to hydration of the flour particles, increase in protein solubility, and disaggregating and reorientation of glutenin polymers to form a membrane network. A large content of high molecular glutelins in the gluten and adequate mixing are important prerequisites for the formation of highly stretchable membranes, capable of retaining many small gas bubbles. Stabilization of the bubbles is also due to the viscosity-building and surface-active components of the aqueous phase of the dough, especially pentosans and polar lipids of the flour. This provides for the required porosity and large baked loaf volume. Numerous and uniformly distributed holes in the crumb, of a diameter of 1–2 mm, contribute to the desirable texture and visual appearance of bread.

A key role necessary for the formation of the required structure of the dough and the backed loaf have intrapeptide and interpeptide disulfide bonds. In the presence of soluble small proteins and low molecular thiol compounds, some of the disulfide cross-links are mobile due to disulfide interchange reactions (Figure 14.26). Oxidizing agents added to the dough improve the texture of bread by promoting the formation of disulfide cross-links. Baking increases the number of disulfide bonds, adding to the stability of the loaf structure. Ascorbic acid, although a reducing agent, added to the dough increases the elasticity and bubble formation, which results in larger loaf and better texture. Other covalent bonds contribute to the properties of the dough. Cross-linking may be caused by dehydroascorbic acid and by the products of its thermal degradation, especially methyl glyoxal,

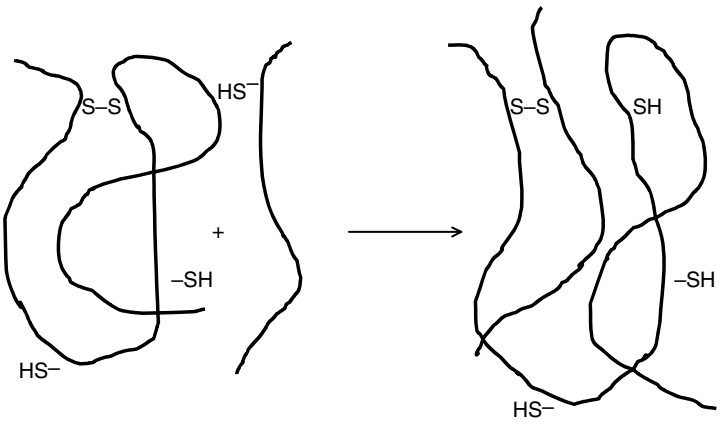

FIGURE 14.26 Interchain thiol and disulfide group reactions.

glyoxal, diacetyl, and threose, which can react with lysine residues of the proteins [33]. Covalent cross-links can also probably result from enzymatic formation of γ-butyraldehyde catalyzed by endogenous diamine oxidase and subsequent addition of nucleophilic amino acid residues [34]. Hydrophobic interactions in aqueous systems between the nonpolar amino acid residues of the flour proteins also contribute to buttressing the dough structure, especially during baking of bread, since their energy increases with temperature. The role of ionic repulsion/attraction forces, albeit not numerous because of the considerably small number of ionizable residues in cereal proteins, may also contribute, since their energy is only slightly lower than that of covalent bonds [7,35].

Hydrogen bridges significantly affect the structure of the dough too. According to Lefebvre et al. [36] gluten forms a network due to aggregation of particles primarily under the effect of hydrogen bonds and hydrophobic interactions, with the disulfide bridges probably not involved directly in the network formation. Although hydrogen bonds are much weaker than covalent bonds, they may be formed in large numbers due to the high content of glutamine residues in cereal proteins and numerous hydroxyl groups in starch and pentosans. Interactions between proteins and starch also contribute significantly to the rheological properties of the system [37]. The interchange reactions of the hydrogen bonds under mechanical stress facilitate stress relaxation of molded dough.

The hydrothermal changes of starch granules described in Section 14.7 also affect the texture of the dough and bread. The degree of swelling of starch depends on the availability of water in the dough in the presence of the competing, very hydrophilic prolamins, glutelins, and pentosans. In case of insufficient swelling the crumb tends to crush; too much swelling results in a slack-baked bread. At a temperature of about 60°C, the viscosity of the dough starts increasing due to denaturation of the flour proteins and starch changes. The crystalline regions of amylopectin become amorphous at this temperature, while the amylose–lipid complexes are stable up to 110°C, which is never achieved in the crumb. Thus, the changes in the presence of expanding air and volatile fermentation products lead finally to the formation of a porous protein–starch structure. The dough, which can be regarded as foam, turns at the end of baking into a sponge-like crumb of baked bread due to rupturing of the proteinaceous membranes caused by increased internal pressure.

The role of lipids in the formation of the texture of bread is due to their interactions with proteins and starch in the loaf. The spreading of lipids at the air–water interface, facilitated by the lipid-binding proteins, has been described in Section 14.6. The lipids of the flour as well as the lipidic surface-active additives interact with amylose and amylopectin as presented in Section 14.8.

Storage of the loaf leads to retrogradation of starch and staling of bread. The crumb gradually turns firmer, less elastic, and dry. The rate of staling is greatest at about 14°C and is negligible below −5°C and at about 60°C. It can be decreased by increasing the contents of proteins and pentosans in the dough and by emulsifiers, especially monoacylglycerols. Furthermore, bacterial α-amylase retards staling by splitting off branched oligosaccharides from the amylopectin molecules, which prevents the formation of crystalline structures [38].

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15 Characteristics of Milk

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CONTENTS

15.1 INTRODUCTION

For the young of mammals, including humans, milk is the first and, foremost, only food ingested for a considerable period of time. With the domestication of animals, it became possible to include milk in the diet of adult humans as well. For much of the world, particularly in the West, milk from cattle (*Bos taurus*) accounts for nearly all the milk processed for human consumption. In the United States, the dairy industry is primarily based on cow's milk. Therefore, this discussion focuses on the properties of bovine milk.

The total production of milk and major processed dairy products for selected years between 1970 and 2002 is given in Table 15.1. Most of the milk produced is processed as fluid or beverage milk (36–46%). However, the consumption pattern of individual products has changed considerably during the past 32 years. In 1970, plain whole milk represented 78% and plain low-fat milk 12% of the beverage milk sold, but in 1992 the consumption pattern was 37% plain whole milk, 46% plain low-fat milk, and 12% plain skim milk. Furthermore, although the amount of beverage milk consumed has remained relatively constant, its percentage of the total milk consumed in all forms has fallen. A noteworthy trend is the large increase in yogurt consumption.

During the past 30 years a new fluid milk product known as ultra-high-temperature (UHT) processed and aseptically packaged milk has appeared in the U.S. market. This product, which is commercially sterile and can be distributed and merchandized without refrigeration, has not yet gained acceptance in the United States; however, it represents a large share of the fluid milk market in many other countries. The biological origin and chemical nature of milk are considered next.

15.2 MILK BIOSYNTHESIS

In the early years of dairy chemistry, the product of the mammary gland was more thoroughly investigated and characterized than the gland itself because of the economic importance of milk products. More recently, however, with advances in molecular and cellular biology, the mammary gland has been the subject of intensive research. Several excellent reviews on milk biosynthesis

TABLE 15.1
U.S. Milk Production and Amounts of Dairy Products for Selected Years Between 1970 and 2002

		Value-Added Products[a]						
Year	Milk Production (kg in billions)	Beverage Milk[b] (kg in billions)	Cheese (kg in billions)	Yogurt (kg in billions)	Dried Milks (kg in billions)	Canned Milk (kg in billions)	Butter (kg in billions)	Frozen Desserts (L in billions)
1970	53.1	24.0	1.5	0.08	0.69	0.58	0.52	4.0
1975	52.4	24.2	1.7	0.20	0.48	0.42	0.45	4.3
1980	58.4	24.1	2.3	0.27	0.57	0.33	0.52	4.3
1985	65.0	24.5	2.7	0.44	0.69	0.29	0.57	4.6
1990	67.4	24.9	3.1	0.47	0.48	0.27	0.59	4.5
2002	77.0	—	3.9	0.97	0.76	0.27	0.62	8.8

[a] Includes all types of cheeses except Cottage cheese.

[b] Includes whole, lowfat, skim, and flavored milks.

Sources: Adapted from U.S. Department of Agriculture (1993). Dairy S&O Yearbook, DS-441, August, Washington, D.C., pp. 1–39 and the USDA, National Agricultural Statistics Service, Dairy Products 2002 Summary.

have appeared and the reader is referred to these for more complete information [32–35,39,48,51]. Some people have argued that animal agriculture will disappear as the human population expands and competition for available grain intensifies. The fallacy of this argument, however, lies in the presence of a rumen in the cow's digestive system. The rumen allows the cow to synthesize nutrients from crude cellulosic and fibrous plant materials and from simple forms of nitrogen, such as urea. Consequently, these animals should be viewed as a unique means of producing high-quality foods from otherwise unusable feedstocks, rather than as inefficient and unnecessary.

The bovine mammary gland, resulting from years of genetic selection, is an amazingly productive organ for biosynthesis. In 1993, the average cow in the United States produced 7055 kg (15,554 lb) of milk in a 305-day lactation [2]. Some high-producing animals yielded as much as 22,700 kg of milk (over 50,000 lb). The cellular machinery of this gland is shown at various levels of magnification in Figure 15.1. Milk originating in the secretory tissue collects in ducts that increase in size as the teat region is approached. The smallest complete milk factory, which includes a storage area, is the alveolus. It is a roughly spherical microorgan consisting of a central storage volume (the lumen) surrounded by a single layer of secretory epithelial cells, which is connected to the duct system. These cells are directionally oriented such that the apical end with its unique membrane is positioned next to the lumen, and the basal end is separated from blood and lymph by a basement membrane. Consequently, a directional flow of metabolites occurs through the cell, with the building blocks of milk entering from the blood through the basolateral membrane. The basic components of milk are synthesized on the production lines of the endoplasmic reticulum, which is supplied with energy by oxidative metabolism in the mitochondria. The components are then packaged in secretory vesicles by the Golgi apparatus or as lipid droplets in the cytoplasm. Finally, the vesicles and lipid droplets pass through the apical plasma membrane and are stored in the lumen. The layer of secretory epithelial cells surrounding the lumen of the alveolus is in turn surrounded by a layer of myoepithelial cells and blood capillaries. When oxytocin, a pituitary hormone circulating in the blood, binds to the myoepithelial cells, the alveolus contracts, expelling the milk stored in the lumen into the duct system.

It is currently thought that the intracellular membranes (endomembranes) also exhibit directional flow to the apical plasma membrane, with concurrent transformation of membranes from endoplasmic reticulum to Golgi, to secretory vesicles, to apical plasma membrane within the epithelial secretory cell. The endoplasmic reticulum, the endomembrane production line where active synthesis occurs, appears to be like a cellular plumbing system, with the inside of these tubes, known as the cisternae, emptying into the Golgi apparatus. The Golgi apparatus transforms into Golgi vesicles, the packages that carry the aqueous phase milk components to the apical plasma membrane.

Ribosomes, the machinery for protein synthesis, exist both free in the cytoplasm and bound to the endoplasmic reticulum. Near the basolateral membrane, the endoplasmic reticulum is covered with ribosomes, making this membrane appear "rough." However, moving in the direction of the apical end, amino acids are depleted and synthesis slows, allowing ribosomes to dissociate. Hence, this membrane becomes smooth as it is transformed into Golgi membrane. Synthesis is completed in the lumen of the Golgi; for example, proteins are glycosylated and phosphorylated and lactose is synthesized. It is here and in the Golgi vesicles that casein micelles first appear. Furthermore, this membrane is impermeable to lactose, so that the major secretory products of the cell are now segregated from all other cellular constituents. Secretion of the products of synthesis is completed when the Golgi vesicles merge with the apical plasma membrane, fuse to become part of that membrane, and empty the contents of the package into the alveolar lumen for storage. Note that in the membrane transformation, the inside or lumen side of the vesicle membrane becomes the outside of the cell plasma membrane. The caseins, β-lactoglobulin, and α-lactalbumin are synthesized in the mammary epithelial cells. Serum albumin and immunoglobulins are not synthesized in these cells, but specific receptors for these proteins appear on the basolateral membrane. Hence, these proteins are transported from blood to the alveolar lumen by internalization of the protein–receptor complex and passage through the cell in membrane vesicles.

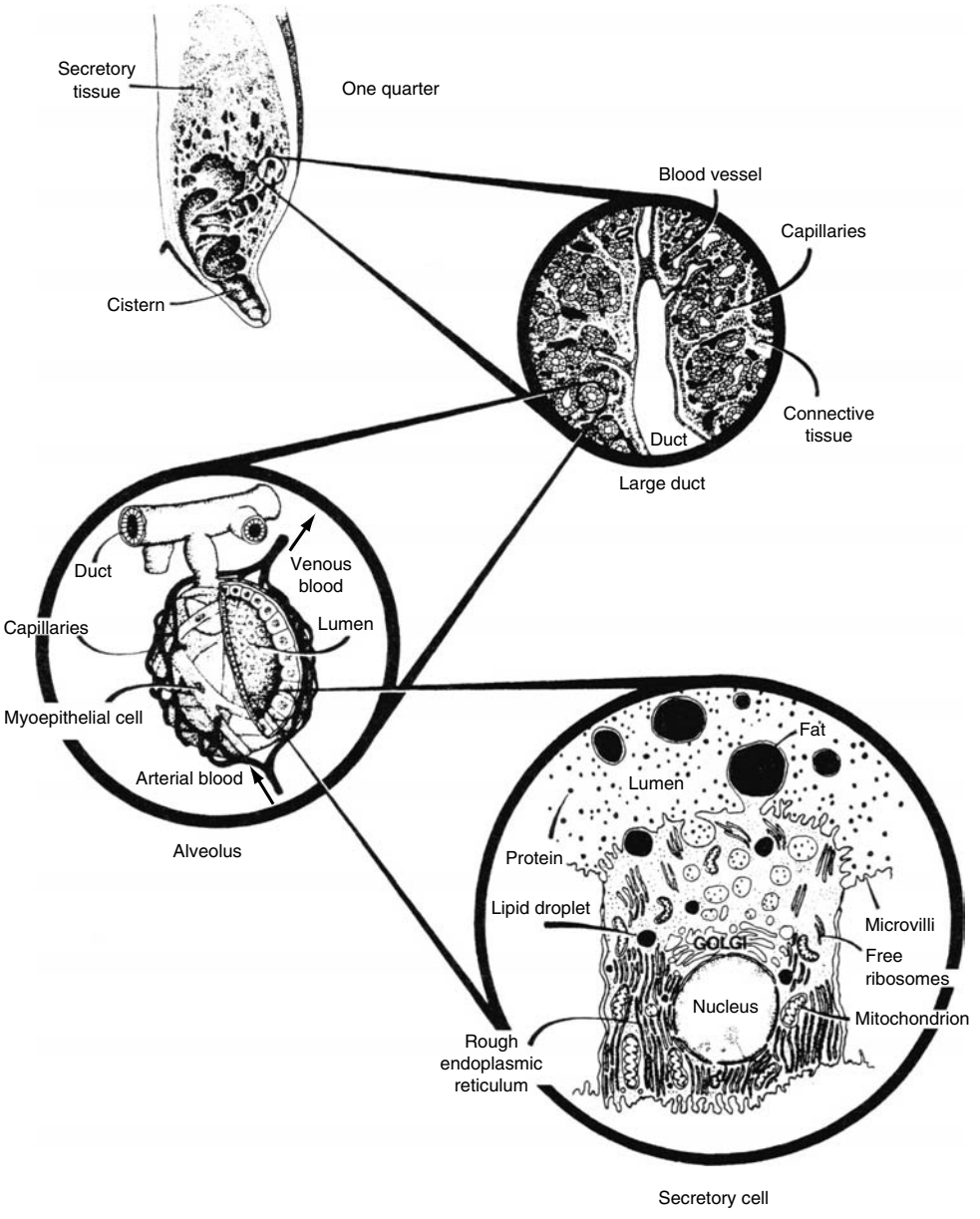

FIGURE 15.1 Bovine mammary gland at increasing levels of magnification.

Fat, which is also synthesized by the endoplasmic reticulum, is directed to the cytoplasmic side of the membrane where it collects as lipid droplets. These droplets move to the apical plasma membrane, where they are expelled into the alveolar lumen by pinocytosis, thus acquiring on their surface a coat of plasma membrane. The presence of this membrane has important consequences for the processing characteristics of milk products. Apical plasma membrane, lost in this process, is continually replenished, at least in part, by the fusion of vesicles as described previously. Electrolytes in milk appear to be transported from blood by pumps or channels in the basolateral membrane. The proportion of sodium and potassium in milk resembles the composition of the cytoplasm. However, because milk is isosmotic with blood plasma and because a large portion of that osmolality is contributed by lactose, the concentration of electrolytes is lower in milk than in the cytoplasm or in plasma.

It should be appreciated that many, if not all, of the characteristics of milk and its constituents are a consequence of the mechanism of synthesis and secretion.

15.3 CHEMICAL COMPOSITION

The composition of milk reflects the fact that it is the sole source of food for the very young mammal. Hence, it is composed of a complex mixture of lipids, proteins, carbohydrates, vitamins, and minerals. In addition, milk contains minor components derived from the cellular synthetic "machinery." The average composition of milk with respect to major classes of compounds and the range of average values for milks of Western cattle are given in Table 15.2. The greatest variability in composition is exhibited by the lipid fraction. Milk composition is influenced both by the diet and the breed. In the past, breeders have selected cows for high-fat production because of the economic value of this constituent. Currently, more importance is being placed on protein content, and breeders are beginning to select for higher protein/fat ratios and higher protein yield.

Because of the major contribution of lactose and milk salts to osmolality, and the required matching of milk's osmotic pressure with that of blood, very little variability is observed in the sum of these constituents. It should be noted that ash does not truly represent milk salts because organic salts are destroyed by ashing; for example, various salts of citrate are principal components of the milk salt system. The pH of freshly drawn milk is 6.6–6.8, which is slightly lower than that of blood.

Most of the constituents of milk are not present as individual molecules in solution. Instead, they exist in large, complex, associated structures (Table 15.3). This is especially true for the caseins, which are present as large spherical micelles, and the lipids, which form even larger spherical globules. Because of the spherical shape and the reduction in the effective number of molecules (kinetic units), both the viscosity and the osmotic pressure are much less than would be observed if these structures were not formed.

15.3.1 Milk Proteins

Milk contains 30–36 g/L of total protein and it rates very high in nutritive quality. There are six major gene products of the mammary gland: α_{S1}-caseins, α_{S2}-caseins, β-caseins, κ-caseins, β-lactoglobulins, and α-lactalbumins (Table 15.4). Each of these proteins exhibits genetic polymorphism because they are products of codominant, allelic, and autosomal genes. Milk proteins

TABLE 15.2
Composition of Bovine Milk from Western Cattle

Component	Average Percentages	Range for Western Breeds[a] (average percentages)
Water	86.6	85.4–87.7
Fat	4.1	3.4–5.1
Protein	3.6	3.3–3.9
Lactose	5.0	4.9–5.0
Ash	0.7	0.68–0.74

[a] Western breeds include Guernsey, Jersey, Ayshire, Brown Swiss, Shorthorn, and Holstein.

Source: Adapted from Corbin, E. A. and E. O. Whittier (1965). In *Fundamentals of Dairy Chemistry* (B. H. Webb and A. H. Johnson, eds.), AVI, Westport, CT, pp. 1–36.

TABLE 15.3
Numbers and Sizes of Major Milk Constituents

Constituent	Size (Diameter, nm)	Number/mL
Lactose	0.5	10^{19}
Whey protein	4–6	10^{77}
Casein micelle	30–300	10^{14}
Milk fat globule	2000–6000	10^{10}

TABLE 15.4
Concentrations of the Major Proteins in Milk

Protein	Concentration (g/L)	Approximate Percentage of Total Protein
Caseins	24–28	80
α_S-Caseins	15–19	42
α_{S1}	12–15	34
α_{S2}	3–4	8
β-Caseins	9–11	25
κ-Caseins	3–4	9
γ-Caseins	1–2	4
Whey proteins	5–7	20
β-Lactoglobulins	2–4	9
α-Lactalbumin	1–1.5	4
Proteose-peptones	0.6–1.8	4
Blood proteins		
Serum albumin	0.1–0.4	1
Immunoglobulins	0.6–1.0	2
Total	100	100

are classified as either caseins or whey proteins. All the caseins exist with calcium phosphate in a unique, highly hydrated spherical complex known as the casein micelle. Such complexes vary in size from 30 to 300 nm in diameter, with a small percentage approaching 600 nm.

Milk proteins can be readily separated into casein and whey protein fractions. Casein comprises 80% of the bovine milk proteins; consequently, curd formed by agglomeration of casein micelles during cheese manufacture retains most of the total milk protein. The other proteins pass into the cheese whey, hence the designation whey protein. Historically, separation of caseins from other proteins by means of precipitation at their isoelectric point (around pH 4.6), yielding so-called acid casein, or by enzyme-induced agglomeration of casein micelles (renneting), as in cheesemaking, has formed the basis for many milk products such as cheese, whey protein products, and other protein ingredients. In the past whey was often discarded; however, it is now economically feasible to concentrate or isolate whey proteins that exhibit excellent functionality and nutritional properties.

In addition to β-lactoglobulin and α-lactalbumin, which are gene products of the mammary gland, whey also contains serum albumin and immunoglobulins that are derived from blood. Milk also contains several "protein" components that are actually large polypeptides. These result from post-translational proteolysis of milk proteins by the indigenous milk proteinase plasmin, which is derived from blood. Thus, γ-caseins that fractionate with the caseins and most of the proteose-peptones, which are present in whey, are derived by limited proteolysis of β-casein.

TABLE 15.5
Amino Acid Composition of the Major Milk Proteins in the Milk of Western Cattle

	Number of Amino Acids in					
Acid	α_{S1}-Casein B-8P	α_{S2}-Casein A-11P	κ-Casein B-1P	β-Casein A^2-5P	β-Lactoglobulin A	α-Lactalbumin B
Nonpolar						
Pro	17	10	20	35	8	2
Ala	9	8	15	5	14	3
Val	11	14	11	19	10	6
Met	5	4	2	6	4	1
Ile	11	11	13	10	10	8
Leu	17	13	8	22	22	13
Phe	8	6	4	9	4	4
Trp	2	2	1	1	2	4
Polar, neutral						
Asn	8	14	8	5	5	12
Thr	5	15	14	9	8	7
Ser	8	6	12	11	7	7
Gln	14	16	14	20	9	5
Gly	9	2	2	5	3	6
Tyr	10	12	9	4	4	4
Cystine	0	1	1	0	2	4
Cysteine	0	0	0	0	1	0
PyrGlu	0	0	1	0	0	0
Polar, acidic						
Asp	7	4	3	4	11	9
Ser P	8	11	1	5	0	0
Glu	25	24	12	19	16	8
Polar, basic						
Lys	14	24	9	11	15	12
His	5	3	3	5	2	3
Arg	6	6	5	4	3	1
Total residues	199	207	169	209	162	123
Molecular weight	23,614	25,230	19,006	23,988	18,363	14,174

Source: Adapted from Swaisgood, H. E. (2003). In *Advanced Dairy Chemistry. 1. Proteins*, 3rd edn., Part A (P. F. Fox and P. L. H. McSweeney, eds.), Kluwer Academic/Plenum Publishers, New York, pp. 139–201.

The amino acid compositions and covalent molecular weights of the major milk proteins are very accurately known from amino acid analyses and from the determination of primary structure by chemical sequencing and by inference from gene sequencing (Table 15.5). A key distinction of the caseins is their large content of phosphoseryl residues and their moderately large proline content. Although the caseins are rather low in half-cystine, whey proteins contain considerably more of this residue.

For a more complete discussion of the characteristics of individual milk proteins, the reader is referred to several excellent reviews [8,9,26,37,38,41–43,59,67–70,72,76,77].

15.3.2 Milk Lipids

Bovine milk contains the most complex lipids known. For detailed characteristics of the various lipids and a discussion of their biosynthesis, the reader should consult one of the reviews [3,7,27–29,51].

TABLE 15.6
Lipid Composition of Bovine Milk

Lipid	Weight Percent	g/L[a]
Triacylglycerols (triglycerides)	95.80	30.7
1,2-Diacylglycerols (diglycerides)	2.25	0.72
Monoacylglycerols (monoglycerides)	0.08	0.03
Free fatty acids	0.28	0.09
Phospholipids	1.11	0.36
Cholesterol	0.46	0.15
Cholesterol ester	0.02	0.006
Hydrocarbons	Trace	Trace

[a] Based on the usual butterfat percentage of commercial pasteurized whole milk, 3.2%.

Source: Adapted from Jensen, R. G., et al. (1991). *J. Dairy Sci.* 74:3228–3243.

TABLE 15.7
Major Fatty Acid Constituents of Bovine Milk Fat

Fatty Acid	Weight Percent	Fatty Acid	Weight Percent
4:0	3.8	16:0	43.7
6:0	2.4	18:0	11.3
8:0	1.4	14:1	1.6
10:0	3.5	16:1	2.6
12:0	4.6	18:1	11.3
14:0	12.8	18:2	1.5
15:0	1.1		

Source: Adapted from Jensen, R. G., et al. (1991). *J. Dairy Sci.* 74:3228–3243 and Timmen, H. and S. Patton (1989). *Lipids* 23:685–689.

Triacylglycerols (triglycerides) represent by far the greatest proportion of the lipids, comprising 96–98% of the total (Table 15.6). In milk, the triacylglycerols are present as globules 2–6 μm in diameter surrounded by membrane material derived from the apical cell membrane. The concentrations listed in Table 15.6 are representative of fresh milk. Some lipolysis occurs during storage, giving higher concentrations of free fatty acids and mono- and diacylglycerols. Triacylglycerols containing three different fatty acids have a chiral carbon at the *sn*-2 position of the glycerol skeleton. Over 400 different fatty acids have been identified in bovine lipids. Consequently, if positional isomers are considered, 400^3 or 64 million triacylglycerol species are theoretically possible. However, only 13 fatty acids are present at concentrations exceeding 1% (w/w) (Table 15.7) corresponding to a theoretical maximum number of isomers of $13^3 = 2197$. If only compositional species, rather than positional isomers, are considered, then the possible number of triacylglycerols with different fatty acid compositions is $n!/3!(n-1)!+n(n-1)+n$, where n is the number of different fatty acids. With 13 different fatty acids, this results in 455 triacylglycerols with different fatty acid compositions. Recently [21], 223 triacylglycerols with different compositions accounting for 80% of the total triacylglycerols were isolated by reversed-phase liquid chromatography and identified. The total mol% of each class of triacylglycerols and eight of the most abundant compositional species (concentrations exceeding 2 mol%) are listed in Table 15.8. Saturated and monounsaturated triacylglycerols comprise the bulk

TABLE 15.8
Composition of Major Triacylglycerols in Milk

Type	Carbon Number	Molecular Species[a]	Mol%
Saturated triacylglycerols (total)[b]			32.4
	34	4:0 14:0 16:0	3.1
	36	4:0 16:0 16:0	3.2
	38	4:0 16:0 18:0	2.5
Monounsaturated triacylglycerols (total)[b]			32.6
	38	4:0 16:0 18:1	4.2
	40	6:0 16:0 18:1	2.0
	48	14:0 16:0 18:1	2.8
	50	16:0 16:0 18:1	2.3
	52	16:0 18:0 18:1	2.2
Diunsaturated triacylglycerols (class 011) (total)[c]			10.6
Diunsaturated triacylglycerols (class 002) (total)[c]			2.5
Polyunsaturated triacylglycerols (all classes) (total)[c]			5.2

[a] Carbon chain length: number of double bonds present. Position of acyl chains in the glycerol skeleton was not determined.
[b] This row gives the total mol% of the specified triacylglycerol type. Sublistings under each type are specific triacylglycerols present in concentrations exceeding 2.0 mol%.
[c] Class 011 indicates two monounsaturated fatty acids in the triacylglycerol, while class 002 indicates one diunsaturated fatty acid present.

Note: 80% of the triacylglycerols were identified in this study; for example, triacylglycerols containing odd-numbered fatty acids were not quantified.

Source: Adapted from Gresti, J. M., et al. (1993). *J. Dairy Sci.* 76:1850–1869.

of the lipids (65 mol%), and the eight most prevalent triacylglycerols represent nearly one-fourth of the total.

A nonrandom distribution of fatty acids in the triacylglycerols is observed. The nonrandomness results from the specificities of the acyltransferase enzymes involved in synthesis. Thus, long-chain acylcoenzyme A is the preferred substrate for the acyltransferase specific for *sn* positions 1 and 2. Conversely, esterification by short-chain fatty acids is much faster with the enzyme specific for the *sn*-3 position. As a result, triacylglycerols with one short-chain and two medium- or long-chain fatty acids are preferentially synthesized, whereas simple or mixed saturated long-chain triacylglycerols are present at low concentrations. These observations are clearly supported by the data in Table 15.9. For example, 85% of butyrate and 58% of caproate are at position *sn*-3, while only 13% of palmitate and 16% of stearate are located in this position.

In addition to exhibiting the greatest percent variability, milk lipids and fatty acids in particular are the most subject of all milk constituents to alteration by environmental factors, such as diet. Because synthesis of short-chain fatty acids and biohydrogenation occurs in the rumen, the ratio of saturated to unsaturated fatty acids does not vary greatly. However, if unsaturated fatty acids are fed in a protected form by encapsulation in denatured protein, they will pass through the rumen unaltered, resulting in greater incorporation of unsaturated acids in the triacylglycerols. Such alteration of triacylglycerol composition is currently attractive because of the reported health benefits of unsaturated fatty acids; however, the possible effects on the oxidative stability of the lipid and the resulting flavor instability have not been fully assessed.

The phospholipid and cholesterol fractions of milk lipids exist in the membrane material introduced into the milk during pinocytosis of lipid droplets through the plasma membrane into the lumen of the alveolus. Lipids of the milk fat globule membrane (Table 15.10), which are similar

TABLE 15.9
Positional Distribution of the Individual Fatty Acids in Bovine Milk Triacylglycerols

	sn Position (mol%)		
Fatty Acid	**1[a]**	**2[a]**	**3[a]**
4:0	5.0 (10)	2.9 (5)	43.3 (85)
6:0	3.0 (16)	4.8 (26)	10.8 (58)
8:0	0.9 (17)	2.3 (42)	2.2 (41)
10:0	2.5 (21)	6.1 (50)	3.6 (29)
12:0	3.1 (25)	6.0 (47)	3.5 (28)
14:0	10.5 (27)	20.4 (54)	7.1 (19)
16:0	35.9 (45)	32.8 (42)	10.1 (13)
16:1	2.8 (48)	2.1 (36)	0.9 (16)
18:0	14.5 (58)	6.4 (26)	4.0 (16)
18:1	20.6 (42)	13.7 (28)	14.9 (30)
18:2	1.2 (28)	2.5 (60)	0.5 (12)

[a] Number in parentheses represents the percent of a particular fatty acid occupying the designated position.

Sources: Jenness, R. (1974). In *Lactation—A Comprehensive Treatise*, vol. III (B. Larson and V. R. Smith, eds.), Academic Press, New York, pp. 3–107; Jensen, R. G., et al. (1991). *J. Dairy Sci.* 74:3228–3243; Patton, S. and R. G. Jensen (1976). *Biomedical Aspects of Lactation*, Pergamon Press, New York; and Pitas, R. E., et al. (1967). *J. Dairy Sci.* 50:1332–1336.

TABLE 15.10
Composition of the Lipid Portion of the Milk Fat Globule Membrane

Component	**Percentage of Membrane Lipids**
Carotenoids	0.45
Squalene	0.61
Cholesterol esters	0.79
Triacylglycerols[a]	53.41
Free fatty acids	6.30
Cholesterol	5.20
Diacylglycerols	8.14
Monoacylglycerols	4.70
Phospholipids	20.40

[a] Contains a large portion of high-melting glycerides (melting point, 52–53°C).

Source: Thompson, M. P., et al. (1961). *J. Dairy Sci.* 44:1589–1596.

to those of cellular plasma membrane, consist of more than 20% phospholipids, the major types being phosphatidylethanolamine (22.3%), phosphatidylcholine (33.6%), and sphingomyelin (35.3%).

Due to a correlation between cholesterol and atherosclerosis, the content of this sterol in foods has received much attention. Milk contains relatively little cholesterol; for example, an 8-oz. glass of whole milk (227 g) contains 27 mg cholesterol. To put this in perspective, one large egg contains

275 mg cholesterol, 10 small shrimp contain 125 mg, and 100 g of freshwater fish contain 70 mg. Because cholesterol occurs in the fat globule membrane, its concentration in a dairy food is related to fat content.

15.3.3 Milk Salts and Sugar

The salts in milk consist principally of chlorides, phosphates, citrates, and bicarbonates of sodium, potassium, calcium, and magnesium [24,54]. Thus, both inorganic and organic salts are present in milk, and these should not be confused with quantities given for "ash," which represents oxides of the minerals resulting from combustion. Milk salt complexes have sizes ranging from those that are ultrafilterable, including free ions and ion complexes, to those that are of colloidal size. Some of the latter type participates in the structure of casein micelles (Table 15.11).

The ultrafilterable species, which are in equilibrium with colloidal forms, may be obtained in the permeate by dialysis or ultrafiltration (UF). Analysis of the latter permeate provided the basis for formulation of a "simulated milk salt ultrafiltrate" [24], which is commonly used to simulate the milk serum phase for research on milk components. The multivalent ions Ca^{2+} and Mg^{2+} exist principally as complexes, including large amounts of Ca-citrate and Mg-citrate and lesser quantities of $Ca(H_2PO_4)_2$ [24]. Thus, only 20–30% of the total Ca and Mg present as ultrafilterable species exist as free divalent cations. For example, milk contains only 2–3 mM free Ca^{2+}. Likewise, most of the citrate is present as complex ions, whereas most of the phosphate exists as $H_2PO_4^-$ and HPO_4^{2-}. Univalent ions such as Na^+, K^+, and Cl^- are present almost entirely as free ions.

Colloidal species of the milk salts bind to milk proteins both as individual ions and as complex structures in casein micelles. These interactions affect the stability and functionality of milk proteins; hence, milk salts play an important role in the properties of dairy foods.

Because of the biosynthetic requirement of isosmolality with blood, one would expect a reciprocal relationship between milk salts and lactose. Such an inverse relationship has been documented between sodium and lactose contents and between sodium and potassium contents [24,54]. Consequently, milk has an essentially constant freezing point (−0.53 to −0.57°C), and this colligative property is used to detect illegal dilution with water.

TABLE 15.11
Concentration and Size of the Principal Salts and Lactose in Milk

	Total in Milk (mg/100 mL)			
Component	**Mean**	**Range of Values**	**Ultrafilterable (%)**	**Colloidal (%)**
Total calcium	121	114–124	33	67
Calcium ion	8	6–16	100	0
Magnesium	12.5	11.7–13.4	64	36
Citrate	181	171–198	94	6
Inorganic phosphorus	65	53–72	55	45
Sodium	60	48–79	96	4
Potassium	144	116–176	94	6
Chloride	108	92–131	100	0
Lactose	4800	4600–4900	100	0

Sources: Davies, D. T. and J. C. D. White (1960). *J. Dairy Res.* 27:171–190; Holt, C. (1985). In *Developments in Dairy Chemistry. 3. Lactose and Minor Constituents* (P. F. Fox, ed.), Elsevier Applied Science, London, pp. 143–181; and White, J. C. D. and D. T. Davies (1958). *J. Dairy Res.* 25:236–255.

Lactose (4-*O*-β-D-galactopyranosyl-D-glucopyranose) is the predominant carbohydrate in bovine milk, accounting for 50% of the solids in skim milk. Its synthesis is associated with that of a major whey protein, α-lactalbumin, which acts as a modifier protein for UDP-galactosyltranferase. Thus, α-lactalbumin changes the specificity of this enzyme such that the galactosyl group is transferred to glucose rather than to glycoproteins. Lactose occurs in both α- and β-forms, with an equilibrium ratio of $\beta/\alpha = 1.68$ at 20°C [49]. The β-form is far more soluble than the α-form, and the rate of mutarotation is rapid at room temperature but very slow at 0°C. The α-hydrate crystal form, which crystallizes under ordinary conditions, occurs in a number of shapes, but the most familiar is the "tomahawk" shape, which imparts a "sandy" mouth feel to dairy products, as in "sandy" ice cream. Lactose, with a sweetness about one-fifth that of sucrose, contributes to the characteristic flavor of milk.

15.3.4 Enzymes

Although present in small amounts, enzymes can have important influences on the stability of dairy foods. Their effects will become even more important as the industry moves toward high-temperature or UHT processing and long storage periods, sometimes at room temperature. These conditions favor survival of enzyme activity or reactivation, and the longer period of storage allows more time for enzyme-catalyzed reactions to occur. The discussion here is limited to enzymes indigenous to milk, but it should be recognized that enzymes are also introduced into milk as a result of microbial growth. The more important enzymes that have been identified are listed in Table 15.12. For a more complete discussion the reader should consult several reviews [17,30,62].

TABLE 15.12
Some Enzymes Indigenous to Bovine Milk[a]

Oxidoreductases
Xanthine oxidase (xanthine:O_2 oxidoreductase)
Sulfhydryl oxidase (protein:peptide-SH:O_2 oxidoreductase)
Lactoperoxidase (donor:H_2O_2 oxidoreductase)
Superoxide dismutase ($O_2^- : O_2^-$ oxidoreductase)
Glutathione peroxidase (GSH:H_2O_2 oxidoreductase)
Catalase (H_2O_2:H_2O_2 oxidoreductase)
Diaphorase (NADH:lipoamide oxidoreductase)
Cytochrome *c* reductase (NADH:cytochrome *c* oxidoreductase)
Lactate dehydrogenase (L-lactate:NAD oxidoreductase)

Transferases
UDP-galactosyltransferase (UDP galactose:D-glucose-1-galactosyltransferase)
Ribonuclease (polyribonucleotide 2-oligonucleotide transferase)
γ-Glutamyltransferase

Hydrolases
Proteinases (plasmin, thrombin, aminopeptidase, and peptidyl peptide hydrolase)
Lipase (gylcerol ester hydrolase)
Lysozyme (mucopeptide *N*-acetylneuraminyl hydrolase)
Alkaline phosphatase (orthophosphoric monoester phosphohydrolase)
ATPase (ATP phosphohydrolase)
N-Acetyl-β-D-glucosaminidase
Cholinesterase (acylcholine acylhydrolase)
β-Esterase (carboxylic ester hydrolase)
α-Amylase (α-1,4-glucan-4-glucanohydrolase)
β-Amylase (β-1,4-glucan maltohydrolase)
5′-Nucleotidase (5′-ribonucleotide phosphohydrolase)

Lyases
Aldolase (fructo-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase)
Carbonic anhydrase (carbonate hydrolase)

[a] The systematic name is given in parentheses.

Sources: Compiled in part from Farkye, N. Y. (1992). In *Advanced Dairy Chemistry. 1. Proteins* (P. F. Fox, ed.), Elsevier Applied Science, London, pp. 339–367; Kitchen, B. J. (1985). In *Developments in Dairy Chemistry. 3. Lactose and Minor Constituents* (P. F. Fox, ed.), Elsevier Applied Science, London, pp. 239–279; and Shahani, K. M., et al. (1973). *J. Dairy Sci.* 56:531–543.

Many of the enzymes are associated primarily with the membranes in milk, such as the fat globule membrane or skim milk membrane vesicles, or with casein micelles. For example, xanthine oxidase, sulfhydryl oxidase, and γ-glutamyltransferase are primarily associated with the membrane fractions, plasmin and lipase are associated with casein micelles, and catalase and superoxide dismutase occur primarily in the milk serum. However, the distribution of enzymes between these constituents is affected by processing and storage conditions, and this can have substantial effects on their activities. Thus, cold-induced lipolysis of milk may be caused by transfer of lipase from micelles to fat globules, and cold storage may cause some dissociation of proteinase from casein micelles. These enzymes can have significant effects on flavor and protein stability in dairy foods. The oxidoreductases can also have effects on flavor stability, particularly in the lipid fraction, because of their influence on the oxidative state.

Milk also contains many vitamins, which are enzyme cofactors or precursors of cofactors, and these are discussed in a later section.

15.4 STRUCTURAL ORGANIZATION OF MILK COMPONENTS

15.4.1 Structure of Milk Proteins

The primary structures of all of the major milk proteins have been determined; thus, our scientific knowledge of milk holds a unique position among foods [8,67–70,76,77]. Moreover, three-dimensional structures of the major whey proteins are also known. Although the relationship between protein structure and food functionality is not well understood, significant progress in this area is occurring. This relationship must be borne in mind by the food scientist while examining the structures of food proteins.

15.4.1.1 The Caseins

Caseins exhibit unique interactions with calcium ions and calcium salts, which may be the most important feature of their physiological and nutritional role. Because of their unique primary and tertiary structures, these proteins undergo post-translational phosphorylation. Such modification results in the formation of anionic clusters in the "calcium-sensitive" caseins, while a single residue is phosphorylated in κ-casein (calcium-insensitive). The calcium-sensitive caseins, α_{S1}-, α_{S2}-, and β-caseins, are so designated because of their extremely low solubilities in the presence of Ca^{2+}. Examples of the primary structure of these anionic clusters in calcium-sensitive caseins are given in Figure 15.2. Binding of Ca^{2+} in the clusters results in the discharge and dehydration of this region, altering the balance of hydrophobic interactions and electrostatic repulsive forces. It is believed that these sequences reside in short exons, allowing their duplication and rapid evolution among

Protein	Charge
α_{S1}-Casein(f 61-70)	
Glu • Ala • Glu • SerP • Ile • SerP • SerP • SerP • Glu • Glu	–12
α_{S2}-Casein(f 5-12); α_{S2}-Casein(f 56-63)	
Glu • His • Val • SerP • SerP • SerP • Glu • Glu	–9
SerP • SerP • SerP • Glu • Glu • SerP • Ala • Glu	–11
β-Casein(f 14-21)	
Glu • SerP • Leu • SerP • SerP • SerP • Glu • Glu	–11

FIGURE 15.2 Anionic clusters of calcium-sensitive caseins.

the calcium-sensitive caseins [69,70]. Most likely, the calcium-sensitive caseins evolved from a common ancestral gene, whereas κ-casein arose from a different gene.

Caseins have not been and apparently cannot be crystallized; therefore, their three-dimensional structures are not known. Nevertheless, their primary structures reveal another unique characteristic of these proteins, namely, the distribution of polar and hydrophobic residues. Clustering of polar residues and hydrophobic residues in separate regions of the primary structure suggests the formation of distinct polar and hydrophobic domains and a resulting amphipathic structure [67–70]. In solutions of the isolated individual protein, both β-caseins and κ-caseins self-associate to form large spherical complexes much like a detergent micelle.

The polar domains of the calcium-sensitive caseins are dominated by the phosphoseryl residues and thus carry a large net negative charge at the pH of milk (Figure 15.2). For example, the 40-residue polar domain of α_{S1}-casein encompassing residues 41–80 exhibits a net charge of -20.6 at pH 6.6, which represents almost the total net charge of the entire molecule (Table 15.13). The N-terminal 40 residues and the C-terminal 100 residues are rather hydrophobic. Although the net charge for the entire molecule of α_{S2}-casein is less than that of α_{S1}-casein, it contains several regions of very high net charge density (Table 15.13 and Figure 15.2). Thus, α_{S2}-caseins are more hydrophilic than other caseins and contain three regions of anionic clusters. Hydrophobic regions are confined to residues 90–120 and 160–207. Furthermore, although the C-terminal region is hydrophobic, it also exhibits a large net positive charge, $+9.5$, while the N-terminal 68-residue region has a net negative charge, -21. Hence, the properties of this protein are very sensitive to ionic strength. β-Caseins, on the other hand, are the most hydrophobic of the caseins. The protein has a small 21-residue N-terminal polar domain with a net charge of -11.5 and a single anionic cluster (Table 15.13 and Figure 15.2). The remainder of the molecule is rather hydrophobic. Hence, this molecule is very amphipathic with a polar domain comprising one-tenth of the total residues in the chain but carrying one-third of the total charge at pH 6.6 (the net charge of this polar domain is nearly equal to that for the entire molecule). The large hydrophobic domain of this molecule makes the overall properties of β-caseins very sensitive to temperature.

TABLE 15.13
Charge Characteristics of the Milk Proteins

Protein	Charge at pH 6.6	Isoionic pH
α_{S1}-Casein B-8P	-21.9	4.94
α_{S2}-Casein A-11P	-13.8	5.37
β-Casein A^2-5P	-13.3	5.14
κ-Casein B-1P	-2.0	5.90
β-Lactoglobulin B	-10.0	5.34
α-Lactalbumin B	-2.6	4.80

Note: The nomenclature of the caseins indicates the genetic variant and the number of phosphoseryl residues. For example, α_{S1}-casein B-8P indicates the genetic variant containing 8 phosphoseryl residues.

Sources: Swaisgood, H. E. (1982). In *Developments in Dairy Chemistry. 1. Proteins* (P. F. Fox, ed.), Applied Science, London, pp. 1–59; Swaisgood, H. E. (1992). In *Advanced Dairy Chemistry. 1. Proteins* (P. F. Fox, ed.), Elsevier Applied Science, London, pp. 63–110; and Swaisgood, H. E. (2003). In *Advanced Dairy Chemistry. 1. Proteins*, 3rd edn., Part A (P. F. Fox and P. L. H. McSweeney, eds.), Kluwer Academic/Plenum Publishers, New York, pp. 139–201.

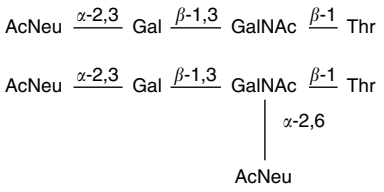

FIGURE 15.3 Trisaccharide and tetrasaccharide structures typical of the carbohydrate moieties attached to κ-casein.

The calcium-insensitive κ-caseins are also very amphipathic with distinct hydrophobic and polar domains; however, the polar domain of κ-caseins does not contain an anionic cluster. κ-Casein is essential to the formation of casein micelle structure. Hence, the physiological functional restraints placed on the structure of this protein are (1) the requirement for interaction with calcium-sensitive caseins to form micelles, thus stabilizing calcium-sensitive proteins in the presence of Ca^{2+}; (2) an amphipathic structure with an inert polar domain that is not precipitated by Ca^{2+}; and (3) a recognition sequence that is specific for limited proteolysis by chymosin, allowing release of the polar domain and coagulation of micelles. Instead of the anionic phosphoseryl clusters characteristic of calcium-sensitive caseins, κ-casein's polar domain contains seryl and threonyl residues that often are glycosylated. This post-translational modification results in attachment of tri- or tetrasaccharide moieties that include anionic *N*-acetylneuraminic acid (AcNeu) residues (Figure 15.3). There are no cationic residues in the C-terminal 53-residue polar domain, and the nonglycosylated domain has a net charge of -11 at pH 6.6; however, κ-casein A-1P(3AcNeu), representing the A variant with one phosphoseryl residue and three AcNeu residues, has a net charge of -14. Because κ-casein does not contain phosphoseryl anionic clusters, it does not bind Ca^{2+} similar to the calcium-sensitive caseins. The polar domain does, however, have many polar amino acids, a large negative charge, and eight evenly spaced prolyl residues creating a highly dehydrated, open, and flexible structure. This polar domain is attached to a large, very hydrophobic domain with many sites that are potentially able to interact with other caseins. Moreover, the Phe—Met peptide bond at position 105–106, which is the N-terminus of the polar domain, is very susceptible to proteolytic cleavage by chymosin, probably because prolyl residues on either side cause these residues to protrude from the surface and increase chain flexibility.

Although three-dimensional structures of the caseins have not been directly determined, global aspects of their structures can be inferred from their physicochemical characteristics and their primary structures. Spectral properties, including circular dichroism (CD) and Raman spectra, as well as predictions based on the primary structures, indicate that all of the caseins possess significant quantities of secondary structure [69,70]. In fact, supersecondary structures have also been suggested such as $\beta\alpha\beta$ motifs in the hydrophobic domain of κ-casein, a turn–β-strand–turn motif centered on the Phe–Met region of κ-casein, and a helix–loop–helix motif with the loop containing the anionic clusters of the calcium-sensitive caseins. Extended secondary structures may also contribute to casein interactions, leading to submicelle formation. However, these structures may be marginally stable and may actually fluctuate during the flexing of the casein chains in solution. Furthermore, physicochemical properties, as well as the proteolytic susceptibilities of isolated caseins, all suggest that their tertiary structures are rather open and flexible. The prevalence of prolyl residues, which interrupt secondary structure and promote turns, may in part be responsible for this openness and flexibility.

15.4.1.2 The Whey Proteins

The structures of β-lactoglobulin and α-lactalbumin are typical of those of other globular proteins [5,6,22,60]. Similar to the caseins, they have a net negative charge at the pH of milk (Table 15.13);

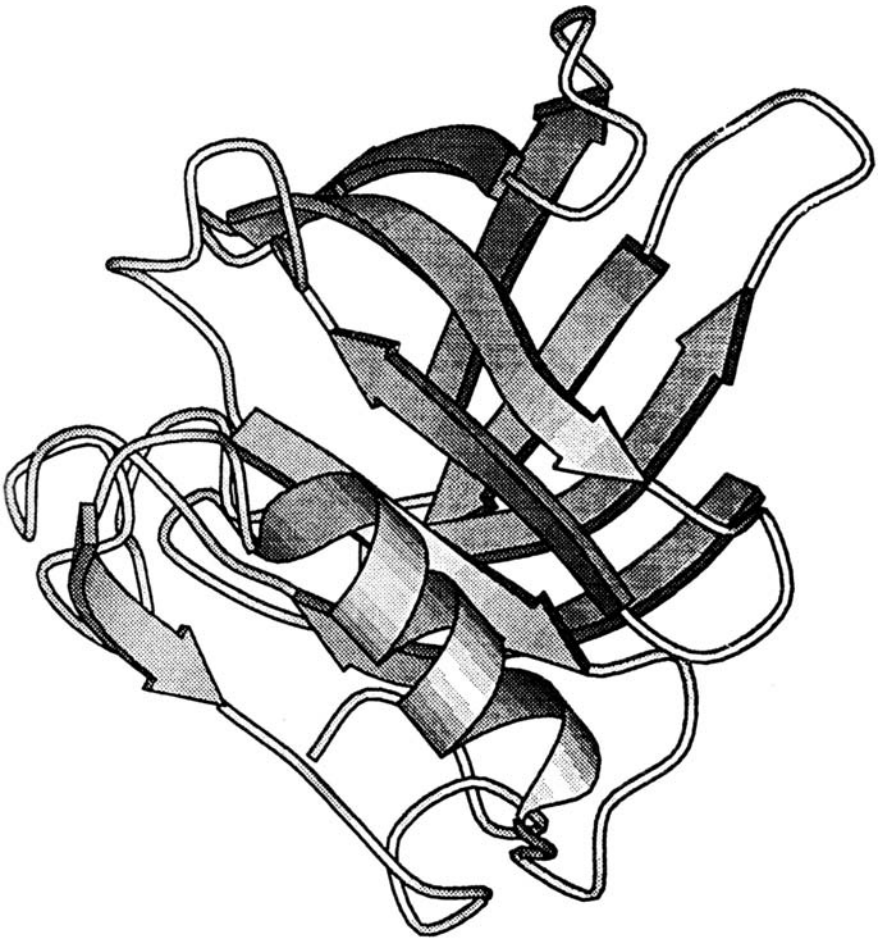

FIGURE 15.4 Three-dimensional structure of β-lactoglobulin. The entrance to the central binding calyx is from the top left, behind the upper β-sheet. This structure was kindly provided by Professor Lindsay Sawyer and was drawn using the MOLESCRIPT program developed by Kraulis [31].

however, unlike the caseins, the sequence distribution of hydrophobic, polar, and charged residues is rather uniform. Consequently, these proteins fold intramolecularly, thereby burying most of their hydrophobic residues so that extensive self-association or interaction with other proteins does not occur. Their three-dimensional structures have been determined by x-ray crystallography and are shown in Figures 15.4 and 15.5.

The tertiary structure of β-lactoglobulin contains a β-barrel structural motif, similar to that of retinol-binding proteins, and a single short α-helix lying on its surface (Figure 15.4). The center of the β-barrel forms a hydrophobic pocket, and a pocket may also exist on the surface between the α-helix and the β-barrel. As a result, β-lactoglobulin binds many small hydrophobic molecules with varying affinities.

The functionality of β-lactoglobulin is governed by these hydrophobic pockets and surface patches, and perhaps even more importantly, by the presence of disulfide bonds and a partially buried sulfhydryl group. Thus, under appropriate conditions this protein readily participates in sulfhydryl–disulfide interchange reactions with itself or with other proteins such as κ-casein.

The interactions of β-lactoglobulin are dependent on pH. At the pH of milk it forms dimers with a geometry resembling two impinging spheres, below pH 3.5 the dimer dissociates to a slightly expanded monomer, between pH 3.5 and 5.2 the dimer tetramerizes to give an octamer, and

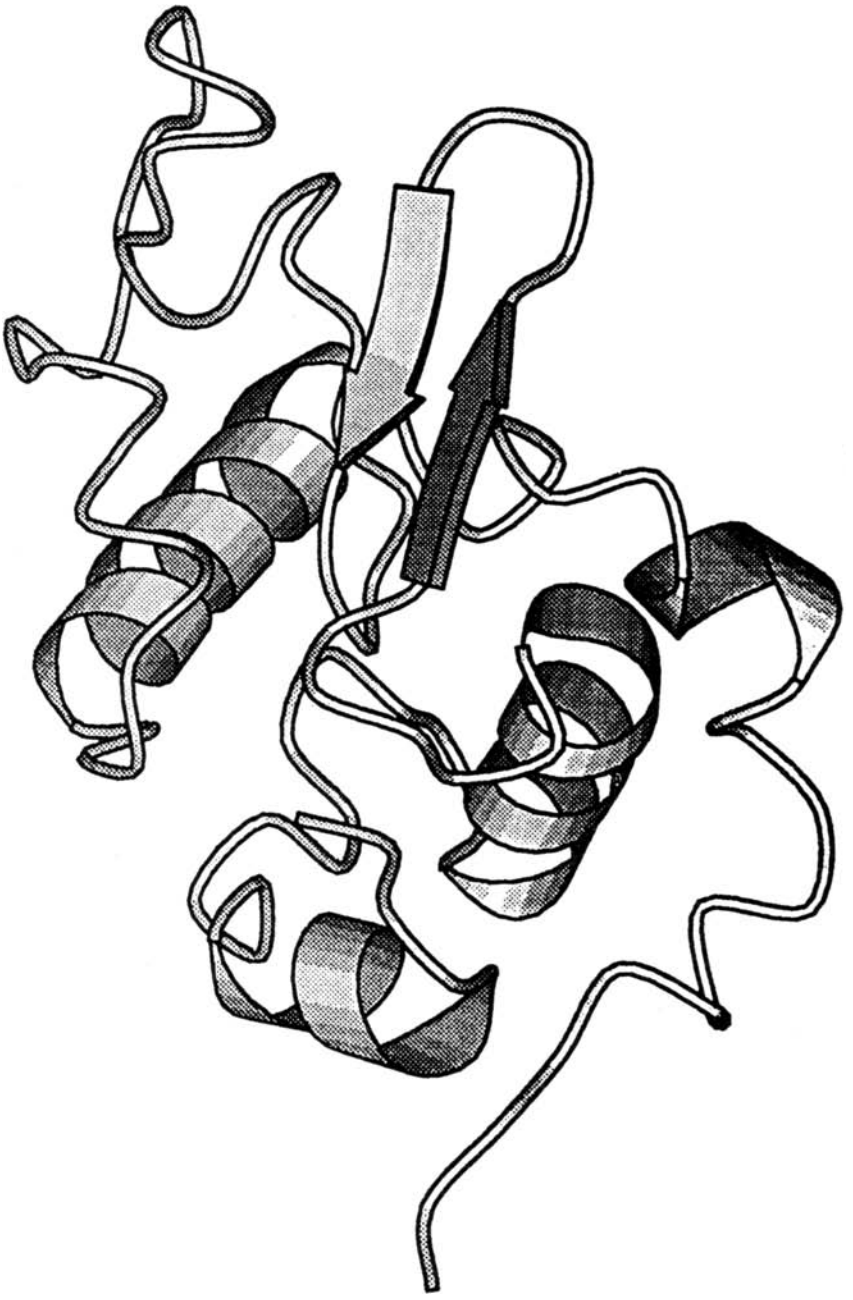

FIGURE 15.5 Three-dimensional structure of α-lactalbumin. This structure was kindly provided by Professor Lindsay Sawyer and was drawn using the MOLESCRIPT program developed by Kraulis [31]. The coordinates used were obtained from the Brookhaven Protein Bank [4] and represent the crystal structure of α-lactalbumin from baboon milk.

above pH 7.5 the dimer dissociates with a concomitant conformational change to give an expanded monomer.

The structural stability of β-lactoglobulin is such that irreversible structural changes occur in the temperature range of many thermal processes for milk and dairy foods; hence, its functionality is very sensitive to precise control of these treatments.

Three-dimensional structure of α-lactalbumin (Figure 15.5) is very similar to that of lysozyme [5,6]. It is a very compact, nearly spherical globular protein containing four α-helices, several 310-helices, and an antiparallel β-sheet. The recent discovery that α-lactalbumin is a calcium metalloprotein has identified the most unique aspect of its structure; thus, a single Ca^{2+} is bound with high affinity in a "calcium binding elbow" composed of 10 residues in a helix–turn–helix motif with coordinating oxygens provided by side-chain carboxyls of Asp 82, 87, and 88 and by peptide carbonyl groups of Lys 79 and Asp 84. *In vitro* folding studies have indicated that the reduced protein, which when oxidized contains four disulfide bonds, does not fold properly in the absence of Ca^{2+}. Also, the holoprotein has less surface hydrophobicity than the apoprotein. Hence, the Ca^{2+} binding is believed to assist protein folding *in vivo* and to aid release of the protein from the endoplasmic reticulum.

With the disulfide bonds intact, as the protein occurs in milk, the tertiary structure unfolds and refolds reversibly. Although α-lactalbumin denatures at a lower temperature than β-lactoglobulin, the transition is reversible except at very high temperatures. Thus, α-lactalbumin, unlike β-lactoglobulin, is not irreversibly thermally denatured under most milk processing conditions.

Physiologically, α-lactalbumin is known to function as a modifier protein of galactosyltransferase, converting it to lactose synthase [5,6]. Its role as a K_M modulator is performed by reversibly binding to the catalytic domain of the integral membrane enzyme galactosyltransferase, which protrudes into the lumen of the Golgi. In the absence of α-lactalbumin, this enzyme transfers galactose from UDP-galactose to form a β-linkage with the 4-hydroxyl group of 3-linked *N*-acetylglucosamine in N-linked oligosaccharides of glycoproteins. When α-lactalbumin is bound, however, the specificity for glucose is increased 1000-fold by reducing the K_M for this substrate. Consequently, lactose is synthesized at the physiological concentrations of glucose in the Golgi.

15.4.2 Casein Micelles and Milk Salts

As a result of their phosphorylation and amphiphilic structure, caseins interact with each other and calcium phosphate to form large spherical micelles of varying size (Table 15.3) with mean diameters of approximately 90–300 nm. Light scattering by these complexes is responsible for the white appearance of milk. A considerable amount of evidence suggests that micelles are formed by association of nearly spherical submicelles having diameters of 10–20 nm. Early electron micrographs of casein micelles suggested a raspberry-like appearance; however, more recent micrographs have questioned this feature (Figure 15.6). Micelles contain 92% protein, composed of α_{S1}:α_{S2}:β:κ-caseins in an approximate mole ratio of 3:1:3:1, and 8% milk salts composed primarily of calcium phosphate but also significant amounts of Mg^{2+} and citrate. The characteristics of micelles determine the behavior of milk and milk products during industrial processes and storage; therefore, the properties of natural micelles and model micelle systems have received considerable study [13,15,52,57,61,64–66].

Micelles have a porous, "spongy" structure with a large voluminosity, approximately 4 mL/g of casein, and exceptional hydration of 3.7 g H_2O/g casein. This hydration is in the order of magnitude larger than that of typical globular proteins. Hence, large molecules, even proteins, have access to and can equilibrate with all parts of the micelle structure. All components of the micelle apparently are in slow equilibria with milk serum. Thus, under appropriate conditions, various caseins and milk salts can be reversibly dissociated from the micelle. Surprisingly, such dissociation may occur to a limited extent without any apparent change in micelle size. Lowering the temperature to near 0°C causes some β-casein, κ-casein, and colloidal calcium phosphate to reversibly dissociate. However, at physiological temperature the amount of individual caseins or submicelles in the serum is extremely small.

Because the structure of the micelle has not been directly determined, the precise location of individual caseins is not known. Nevertheless, all evidence points to a predominant surface location for κ-casein, while α_S- and β-caseins are predominant in the interior. The distribution is not exclusive, however, because the calcium-sensitive caseins are also accessible on the surface. Thus, the amount of κ-casein in the micelle increases linearly with the surface/volume ratio, whereas the amount of

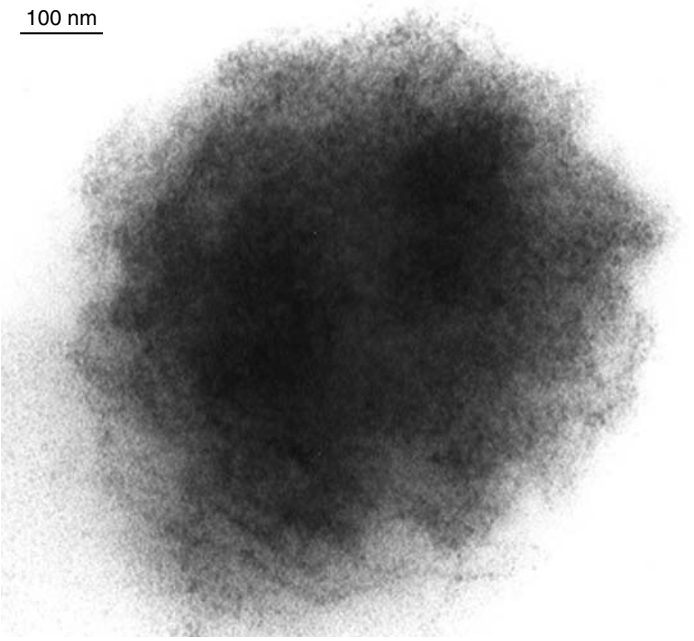

FIGURE 15.6 Electron micrograph of a casein micelle kindly provided by Professor Donald J. McMahon of Utah State University.

β-casein decreases linearly. (*Note:* The surface/volume ratio for a sphere increases with decreasing size.) Because serum components slowly equilibrate with the micelle, the addition of κ-casein causes a decrease in micelle size, while the addition of β-casein causes micelle size to increase. A variable composition of the submicelle, in submicelle models, is supported by these and other observations, such that submicelles rich in the calcium-sensitive caseins have a predominately interior location.

A fundamental characteristic of micelles is their resistance to irreversible association on close approach; for example, pellets formed by sedimentation redisperse spontaneously on standing. Hence, the micelle surface must be highly solvated and unreactive, a key to its stability under many harsh processing conditions.

Recently, a "hairy" layer has been identified on the surface of the micelle, and this layer is presumed to represent the polar domain of κ-casein. The presence of this flexible, highly hydrated polar polypeptide chain provides an inert surface and steric stabilization to the micelle. Cleavage of this domain from κ-casein by chymosin and release of this macropeptide to the milk serum completely changes the surface characteristics of the micelle, causing its destabilization. The modified surface is very active, leading to the association of micelles and formation of gels, such as those in cheese.

Calcium phosphate is the other key component required for the formation of natural casein micelles [24,57]. Although micelle-like complexes can be formed with just Ca^{2+}, inorganic phosphate is required to provide temperature stability. The structure of colloidal calcium phosphate, composed of Ca, inorganic phosphate, Mg, and citrate in fairly fixed proportions, has long been a subject of controversy. Recent x-ray absorption and infrared spectroscopy studies have shown that its structure in micelles closely resembles that of brushite, a crystalline form of $CaHPO_4 \bullet H_2O$ [24]. Ester phosphates in the anionic clusters of caseins may be an integral part of the calcium phosphate–Mg^{2+} citrate matrix.

Various models of micelle structure have been proposed to account for experimental observations and stimulate further investigation. In general, these can be classified as core coat, internal

structure, and subunit models [15,57]. The first subunit model was proposed by Morr [78]. This model was modified by Slattery and Evard [66], by Schmidt [61], by Walstra [79], and finally by Holt [80]. Current thinking based on all available observations favors a subunit model in which the submicelles have a varying composition. Hydrophobic interactions are the predominant force binding the individual caseins together in the submicelle. This may occur through specific interactions between β-sheet structures in the hydrophobic domains of κ-casein and the calcium-sensitive caseins. Hydrophobic interactions cannot be the only force holding the submicelle together because they do not dissociate at low temperatures where such interactions are greatly weakened. A nonuniform distribution of κ-casein in the submicelles allows for interior submicelles rich in calcium-sensitive caseins and surface submicelles rich in κ-casein. When the polar domain of κ-casein, which cannot interact with calcium phosphate, occupies a sufficient amount of the micelle surface area, micelle growth stops; thus, a larger number of submicelles rich in κ-casein yields smaller micelles. In this model, the submicelles interact by incorporation of the anionic phosphoseryl clusters into the brushite-like structure of the colloidal calcium phosphate. However, the relative importance of the salt bridge and protein–protein interactions between submicelles is not clear. It appears that the salt bridge interaction between submicelles is the predominant one; however, the relative importance is probably dependent on temperature.

Recent studies have discovered the formation of calcium phosphate nanoclusters in the presence of the polar domain of β-casein (β-casein(f 1–25)) [15]. The nanoclusters form spontaneously *in vitro* and have a weight of 61 kDa and properties similar to those of micellar calcium phosphate. These observations led to the proposal of a nanocluster model for the micelle that is not composed of subunits but rather a homogeneous matrix of protein with calcium phosphate nanoclusters distributed randomly throughout the micelle and stabilized by ligands contributed by the polar domains of the calcium-sensitive caseins. An attractive feature of this model is its basis on the known biological function of caseins to stabilize the high concentrations of calcium salts in milk.

15.4.3 The Fat Globule

To lessen the surface free energy of lipids in an aqueous medium, the lipid molecules associate to form large spherical globules, thereby minimizing the interfacial area. Milk fat globules are the largest particles in milk (Table 15.3), ranging in diameter from 2 to 6 μm. Light scattering by these large globules is responsible for the "creamy" appearance of whole milk. Fat globules in milk have a mean diameter approximately 25-fold larger than the mean diameter of casein micelles. During secretion of fat globules through the plasma membrane, the globules acquire a coat of the plasma membrane, which serves to stabilize the oil-in-water emulsion [40]. This membrane coat contains cell membrane proteins, including the enzymes, and nearly 70% of the phospholipid and 85% of the cholesterol in milk. Many of the enzymes having activities of importance to milk properties are associated with the fat globule membrane. These include alkaline phosphatase, xanthine oxidase, 5′-nucleotidase, sulfhydryl oxidase, and phosphodiesterase.

The milk fat globule therefore consists of a lipid core composed almost exclusively of triacylglycerols, a layer of some cytoplasmic proteins adsorbed prior to secretion, and an outer covering of plasma membrane. Apparently, however, the plasma membrane does not completely cover the globule because some of the cytoplasmic proteins also appear in the outer surface.

Because fat globules have a lower density than the aqueous phase of milk, they rise and cause "creaming" of unhomogenized milk. However, creaming occurs much more rapidly than would be predicted from the size of individual fat globules. This increased creaming rate is due to the clustering of globules caused by interaction of membrane proteins with immunoglobulin (IgM) and protein in the skim milk membrane vesicles. The large clusters, as theory predicts, rise much more rapidly than single globules.

15.5 USE OF MILK COMPONENTS AS FOOD INGREDIENTS

15.5.1 Effects of Processing on Milk Components

15.5.1.1 The Fat Phase

Milk fat is utilized in three major forms: (1) homogenized, as in whole milk; (2) concentrated, as in creams obtained by centrifugal separation; and (3) isolated, as in butter fat obtained by churning. Excellent reviews of the fat phase and products derived from it are available [7,28]. Homogenization prevents the fat globules from forming a cream layer. The globules are reduced in size from 3 to 10 μm to less than 2 μm in diameter by forcing the liquified fat through restricted passages under high pressure (−2500 psi) at high velocity. As a result, the surface area is increased five- to tenfold from 2 to 10–20 m^2/g. The newly formed surface exhibits high interfacial free energy and rapidly adsorbs protein by hydrophobic interaction. Thus, casein micelles or submicelles and, to a lesser extent, whey proteins are adsorbed (approximately 10 mg/m^2), and these substances prevent coalescence of the newly formed small globules. As a result, these homogenized fat globules are somewhat like large casein micelles with respect to their surface properties. For example, any operation that will cause micelles to aggregate, such as treatment with chymosin (renneting in cheesemaking), acidification, or excessive heating, will also cause these fat globules to aggregate. Consequently, fermented dairy foods made with homogenized milk have different rheological properties than those of comparable foods made from unhomogenized milk, and the heat stability of homogenized cream is less than that of unhomogenized cream and is, therefore, more prone to feathering in coffee.

The major benefit of homogenization is the prevention of creaming. This results from a reduction in fat globule size and a decreased tendency for fat globule coalescence. Resistance to coalescence occurs because of the previously mentioned adsorption of casein micelles, casein submicelles, and whey proteins to the fat globule surface, and the denaturation of immunoglobulins and disruption of skim milk membrane vesicles. A secondary benefit of homogenization is increased whiteness that occurs because the increased number and smaller size of fat globules scatters light more effectively.

Homogenization also has several disadvantages in addition to the previously mentioned reduced heat stability of homogenized cream as compared with unhomogenized cream. Homogenized milk has a blander flavor, fat globules are more susceptible to light-induced oxidation and to lipolysis when active lipase is present, and protein gels have a lower curd tension.

Cream can be obtained by centrifugal separation of cold (5–10°C) or warm unhomogenized milk. Cold separation is less disruptive of the fat globule membrane, and the resulting cream contains more immunoglobulins and is more viscous than cream obtained at a higher temperature. Churning of cream causes disruption of the fat globule membrane, resulting in clumping and coalescence of the exposed triacylglycerol globules. About 50% of the membrane is released into the buttermilk phase during churning. Clumping and coalescence of newly exposed fat globules does not occur properly when the fat is either all solid or all liquid; therefore, the temperature of churning must be carefully controlled to give an appropriate ratio of solid to liquid fat. The churning process causes a phase inversion from an oil-in-water emulsion to a water-in-oil emulsion (butter). The continuous fat phase contains some intact fat globules amounting to 2–46% of the total fat. Butter also contains 16% water as finely dispersed droplets.

15.5.1.2 The Protein Micelle and Milk Salt System

Conditions imposed by processing, such as changes in temperature, pH, and concentration, alter equilibria within the salt system, within the protein system, and between salts and proteins. Often these changes are not completely reversible even if the original conditions are restored, so the final characteristics of a dairy food depend on the processing conditions.

The milk serum is saturated or supersaturated with respect to various calcium phosphates and calcium citrate; consequently, small changes in environmental conditions cause significant shifts in these equilibria [24]. Micellar casein is much less stable than sodium caseinate. In general, changes that decrease micelle voluminosity or increase micellar calcium phosphate will cause a decrease in stability. For example, micelles become less stable in the presence of alcohol or with an increase in Ca^{2+} activity. Pasteurization (71.7°C for 15 s) or UHT processing (142–150°C for 3–6 s) irreversibly increases the amount of colloidal calcium phosphate at the expense of both soluble and ionized calcium and soluble phosphate. Consequently, the pH also decreases, due to release of protons from primary and secondary phosphates. The calcium transformed to tertiary calcium phosphate does not come entirely from the serum because heating also causes dissociation of calcium bound to protein. Thus, pasteurization, and especially sterilization, affects the size distribution of micelles, leading to an increase in the abundance of both large and small micelles. Cooling to 0–4°C has the opposite effect, causing an increase of soluble calcium and phosphate and an increase in pH. As noted previously, some β- and κ-casein also dissociates from the micelle upon cooling. However, these changes induced by cooling are largely reversible.

Individual milks have been classified according to their pH-dependent heat stability (duration of heating required for protein coagulation) [63]. Type A milk exhibits an optimum in the stability curve between pH 6.6 and 6.9, while Type B milk steadily increases in stability as the pH is raised above pH 6.6. This phenomenon appears to be related to the ratio of β-lactoglobulin to κ-casein and the heat-induced interaction between these two proteins. Addition of κ-casein will convert a Type A milk to Type B, and the reverse can be achieved by the addition of β-lactoglobulin to Type B milk.

Pasteurization or UHT processing causes partial, irreversible unfolding of β-lactoglobulin, thereby exposing hydrophobic surface and the lone sulfhydryl group. The subsequent interaction with κ-casein, stabilized by a sulfhydryl–disulfide interchange, alters the surface properties of casein micelles. Preheating unconcentrated milk at 90°C for 10 min reduces its stability to subsequent treatments at higher temperatures. However, the same treatment, when applied to concentrated milk, nearly doubles the ability of the micelles to resist coagulation during subsequent processing at higher temperature. Concentrating milk lowers pH and increases both Ca^{2+} activity and the concentration of colloidal calcium phosphate, thus lowering micelle stability. In concentrated milk, the destabilizing effect of micelle interaction with denatured β-lactoglobulin is more than compensated for by heat-induced reduction of Ca^{2+} activity. Thus, concentrated milks cannot be sterilized without a preheat treatment.

Heat treatments of increasing severity are accompanied by increased production of dehydroalanyl residues, due to β-elimination of disulfide bonds and phosphoseryl residues (see Chapter 5), increased deamidation of asparaginyl and glutaminyl residues, and increased Maillard browning. Cross-linking of protein during heating can result from the reaction of dehydroalanyl residues with ε-amino groups of lysyl residues to form lysinoalanine, or reaction with sulfhydryl groups of cysteinyl residues to form lanthionine. Continued heating (e.g., 20–40 min at 140°C) destabilizes micelles, leading to gel formation. Micelle destabilization results from a combination of factors, the most important being a decrease in pH. This decrease is caused by degradation of lactose, a shift in equilibria from primary and secondary phosphates to hydroxyapatite, and hydrolysis of phosphoserine [19]. The shift in calcium phosphate equilibria occurs rapidly, lowering the pH from about 6.7 to 5.5. Lactose degradation and phosphoserine hydrolysis continue more slowly, yielding an estimated pH of 4.9 at the point of milk coagulation. The colloidal calcium phosphate that forms upon heating is not the brushite-like calcium phosphate–magnesium citrate that occurs in natural micelles; consequently, this change in salt equilibria is mostly irreversible. It should be noted that at pH 4.9 the original colloidal calcium phosphate would be completely dissolved and the high temperature (140°C) and declining pH would cause progressive dissociation of the micelle. Thus, the coagulum that eventually forms most likely consists of a matrix of interacting individual protein chains or submicelles, rather than whole micelles as in rennet curd. Sulfhydryl–disulfide interchange and covalent cross-linking may contribute to the stability of such protein–protein interactions.

Addition of salts, acids, or alkalis to milk affects Ca^{2+} activity and hence the calcium phosphate equilibria. This alters the binding of Ca^{2+} and colloidal calcium phosphate to the micelle, which changes micelle stability. For example, the addition of secondary phosphate stabilizes the micelle system by (1) increasing pH, which increases micellar net charge; (2) reducing the soluble calcium concentration by shifting the equilibria toward tertiary calcium phosphate; and (3) competitive displacement of micellar calcium by sodium. Consequently, this salt may be used as a milk stabilizer. Likewise, polyvalent anions, such as citrate or polyphosphates that bind and increase micellar net charge and lower soluble calcium are excellent milk stabilizers. Conversely, the addition of calcium salts increases Ca^{2+} activity and hence colloidal calcium phosphate, which results in a lower pH and less stable micelles. As noted previously, concentration of milk has a similar effect because the salt equilibrium shifts toward colloidal calcium phosphate and the pH falls; for example, 11-fold concentration of milk increases the soluble calcium and phosphate concentration only eightfold, and the Ca^{2+} concentration only sixfold, while the pH is lowered from 6.7 to 5.9.

These principles must be taken into account in the processing of milk and in the incorporation of milk into formulated foods. Because of the instability of micelles in concentrated milk, stabilizers are usually added prior to concentration. Also, milk is preheated prior to concentration or drying to shift the calcium phosphate equilibria (thereby reducing Ca^{2+} activity) and to promote micelle interaction with denatured β-lactoglobulin. On the other hand, curd-setting properties of milk can be improved by adding calcium salts.

15.5.1.3 The Whey Proteins

Because denaturation of whey proteins occurs rapidly at temperatures above 70°C, normal commercial heat treatments denature at least a portion of these proteins. Major whey proteins exhibit thermostability to structural unfolding in the order α-lactalbumin < albumin < immunoglobulin < β-lactoglobulin. However, thermal unfolding of α-lactalbumin is reversible so that denaturation as measured by irreversible changes indicates an order of increasing thermostability of IgG < serum albumin < β-lactoglobulin < α-lactalbumin. Denatured whey proteins, particularly β-lactoglobulin, are considerably less soluble and more sensitive to precipitation by calcium ions than are their native counterparts. Hence, denatured whey proteins become partially incorporated into cheese curd; however, extensive binding of β-lactoglobulin to micellar κ-casein interferes with chymosin-catalyzed curd formation, so conditions of cheese making must be carefully chosen.

As noted previously, heat-induced association of whey protein (especially β-lactoglobulin) with casein micelles alters micelle properties and increases heat stability. The functionality of whey proteins is very sensitive to the extent of denaturation. For example, if whey proteins have not been heat-denatured they are quite soluble at acid pH, a characteristic that facilitates their incorporation into carbonated beverages. The extent of denaturation is also extremely important and thus must be carefully controlled to optimize their performance as fat mimetics.

15.5.1.4 Lactose

Reaction of the aldehyde group of lactose with the ε-amino group of lysine (onset of Maillard reactions) occurs even under very mild heat treatment, and the reaction continues slowly during storage. The degree of Maillard reaction and attendant browning is very sensitive to the severity of heat treatment, and the effects are desirable in some products and undesirable in others. Very severe heat treatments, such as those used for an in-can sterilization of concentrated milks, causes lactose to partially degrade via Maillard reactions to yield organic acids, principally formic acid. This source of acidity is one of the major causes of protein destabilization in such products.

The very low solubility of the α-anomer of lactose can result in its crystallization in frozen products. Crystallization of α-lactose in frozen milk is typically accompanied by prompt destabilization and precipitation of casein, the reasons for which are not fully understood. Soluble lactose

may have a direct stabilizing effect on casein, and if so, this is lost with lactose crystallization. Lactose crystallization may also have an indirect effect on casein stability, because its removal from the unfrozen phase decreases solute concentration, resulting in additional ice formation. The effect is similar to that observed during concentration of milk. Thus, calcium ion concentration in the unfrozen phase increases and more tertiary calcium phosphate precipitates, yielding a decline in pH and casein instability.

A common defect in ice cream, known as sandiness, is caused by the formation of large α-lactose crystals. This defect has been largely overcome by the use of stabilizers of plant origin that impede formation of lactose nuclei.

15.5.2 Use in Formulated Foods

15.5.2.1 Functionality of Proteins

Proteins from traditional sources are being increasingly utilized as ingredients in a growing number of formulated foods. The benefits of milk proteins as ingredients in other foods stem from their excellent nutritional properties and their ability to contribute unique and essential functional properties to the final foods [16,44,45,47]. The functional properties of a food protein are obviously related to its structure; however, the relationship is currently not understood in sufficient detail to allow the design of protein structure to achieve a specific functionality. Nevertheless, some general features of the relationship between structure and functionality can be outlined.

Protein functional properties are related to several general molecular characteristics such as hydration, surface activity, and the type of protein–protein interactions favored by partially unfolded structures. The functional characteristics associated with these molecular properties are listed in Table 15.14. Hydration is a particularly important parameter because solubility is a requirement for many excellent functional properties and water binding is an essential function in many foods. Surface activity is a complex function of the protein's surface hydrophobicity and its flexibility, which allows it to unfold and spread at an interface. The order of surface activity for various milk protein components is β-casein > monodispersed casein micelles > serum albumin > α-lactalbumin > α_S-casein-κ-casein > β-lactoglobulin [47]. The intermolecular interactions that occur between partially unfolded protein structures are extremely important to functionality, but they are a complex function of protein stability and structure and the type of surface or surface structure exposed in the partially unfolded protein. Caseins are unique because their flexible structures allow interaction with many partially unfolded structures of other proteins by hydrophobic interaction and/or by extension

TABLE 15.14
Relationship Between Protein Molecular Properties and Functionality

Molecular Property	Associated Functional Properties
Hydration	Solubility, dispersibility, swelling, viscosity, gelation, and water absorption
Surface activity	Emulsification, fat adsorption, foaming, and whipping
Protein–protein interactive potential	Aggregation, cohesion, texturization, gelation, elasticity, and extrudability
Molecular structure or architecture yielding organoleptic properties	Color, flavor, and odor

Source: Adapted from Morr, C. V. (1982). In *Developments in Dairy Chemistry. 1. Proteins* (P. F. Fox, ed.), Applied Science, London, pp. 375–399.

of secondary structure. Thermally unfolded proteins appear to be "molten globules" lacking tertiary structure but having secondary structure.

Advances in biotechnology have triggered interest in designing protein functionality for specific applications either by genetic manipulation or by enzymatic modification. Potentially, protein functionality can be altered, and perhaps designed, by specific limited proteolysis. Of course, this is exactly what occurs in the coagulation of milk by chymosin. It is extremely important to carefully control the extent of such reactions to optimize desirable properties and prevent off-flavors. Control of the reaction can be most readily accomplished by using bioreactors containing immobilized enzymes.

Because milk proteins have evolved as the primary source of nutrition for very young mammals, excellent nutritional and flavor properties and freedom from antinutritional factors are ensured.

15.5.2.2 The Caseins

Caseins have excellent solubility and heat stability above pH 6. These proteins also have very good emulsifying properties because of their amphiphilic structure. Example compositions of several types of commercial casein products are given in Table 15.15, and the types of products that use casein as a protein ingredient are given in Table 15.16. The functionality of these commercial products depends on the product type; hence, careful consideration of the requirements for the ingredient must enter into its selection. Four types of casein products are available; acid caseins, rennet caseins, caseinates, and coprecipitates. Roughly 250,000 metric tons of casein products are produced annually worldwide.

Acid (hydrochloric, lactic, and sulfuric) caseins are simply isoelectric precipitates (pH 4.6), and they are not very soluble. Likewise, rennet casein, which is produced by treatment with chymosin (rennet), is not very soluble, especially in the present of Ca^{2+}, because the polar domain of κ-casein

TABLE 15.15
Compositions of Some Casein and Whey Protein Products Commercially Available

Protein Products	Composition (% dry weight)			
	Protein	Ash	Lactose	Fat
Casein products				
Sodium caseinate	94	4.0	0.2	1.5
Acid casein	95	2.2	0.2	1.5
Rennet casein	89	7.5	—	1.5
Coprecipitate	89–94	4.5	1.5	1.5
Whey protein concentrates				
Normal UF WPC	59.5	4.2	28.2	5.1
Neutral UF/DF WPC	80.1	2.6	5.9	7.1
Whey protein isolates				
Spherosil QMA WPI	77.1	1.8	4.6	0.9
Spherosil S WPI	96.4	1.8	0.1	0.9
Vistec	92.1	3.6	0.4	1.3

Sources: Adapted from Morr, C. V. (1982). In *Developments in Dairy Chemistry. 1. Proteins* (P. F. Fox, ed.), Applied Science, London, pp. 375–399 and Morr, C. V. (1989). In *Developments in Dairy Chemistry. 4. Functional Milk Proteins* (P. F. Fox, ed.), Elsevier Applied Science, London, pp. 245–284.

TABLE 15.16
Some Applications of Milk Protein Products in Formulated Foods

Food Category	Specific Food in Which Casein/Caseinates/Coprecipitates are Useful	Specific Foods in Which Whey Proteins are Useful
Bakery products	Bread, biscuits/cookies, breakfast cereals, cake mixes, pastries, frozen cakes, and pastry glaze	Breads, cakes, muffins, and croissants
Dairy-type foods	Imitation cheeses, coffee creamers, cultured milk products, and milk beverages	Yogurt, cheeses, and cheese spreads
Beverages	Chocolate, fizzy drinks and fruit beverages, cream liqueurs, wine aperitifs, and wine and beer	Soft drinks, fruit juices, powdered or frozen orange beverages, and milk-based flavored beverages
Desserts	Ice cream, frozen desserts, mousses, instant pudding, and whipped topping	Ice cream, frozen juice bars, and frozen dessert coatings
Confectionery	Toffee, caramel, fudges, marshmallow, and nougat	Aerated candy mixes, meringues, and sponge cakes
Meat products	Comminuted products	Frankfurters, luncheon rolls, and injection brine for fortification

Source: Adapted from Mulvihill, D. M. (1992). Production, functional properties and utilization of milk protein products, in *Advanced Dairy Chemistry. 1. Proteins* (P. F. Fox, ed.), Elsevier Applied Science, London, pp. 369–404.

has been removed. Rennet casein has a high mineral content because colloidal calcium phosphate–citrate is included in the clotted micelles, whereas, acid casein has a lower mineral content because the calcium phosphate is solubilized and passes into the whey. Coprecipitates of casein and denatured whey proteins are more soluble than acid or rennet casein but are not as soluble as the caseinates. Solubilization of coprecipitates can be achieved by adjustment to alkaline pH and addition of polyphosphates. Because of their low solubility, these products are best suited for products such as breakfast cereals, snack and pasta products, and baked foods, to which they contribute texture and dough-forming characteristics.

The caseinates (sodium, potassium, and calcium) are prepared by neutralizing acid casein with the appropriate alkali prior to drying. These isolates, especially sodium and potassium caseinates, are very soluble and extremely heat stable over a wide range of conditions. Because of their amphiphilic structure, these proteins are useful for emulsification, water binding, thickening, whipping/foaming, and gel formation. Thus, these isolates have found wide acceptance as emulsifiers and water binders in formulated meat products, texturized vegetable protein products, margarine, toppings, cream substitutes, and coffee whiteners.

15.5.2.3 The Whey Proteins

Whey protein concentrates (WPC) or isolates (WPI) are very desirable as nutritional ingredients because of their high concentration of sulfur-containing amino acids compared to plant proteins such as soy proteins. The annual worldwide production of whey protein products is roughly 600,000 metric tons. In the past, these products (lactalbumin (not to be confused with the protein (α-lactalbumin), protein–metaphosphate complex, or protein–carboxymethylcellulose complex) had limited functionality because of extensive protein denaturation or the presence of precipitating reagents. Commercial adoption of membrane technology, including UF and diafiltration (DF), and ion-exchange adsorption (IEA) has resulted in the development of a variety of products with varied and excellent functionalities [46,47]. Three currently used IEA processes are the Vistec process, based on a cellulosic ion exchanger, the Spherosil S process, based on a porous silica cation exchanger,

and the Spherosil QMA process, which uses a porous silica anion exchange material. Compositions of these whey protein products are listed in Table 15.15. Example applications of whey protein ingredients in formulated foods are given in Table 15.16.

Key factors that determine the functionality of a product are the amount of protein denaturation, the lactose content, and the amounts of lipids and minerals present. Generally, protein denaturation lowers the solubility and adversely affects functions requiring high surface activity such as emulsification and foaming. Thus, UF WPC, UF/DF WPC, and Spherosil QMA WPI have excellent solubilities and emulsifying properties consistent with low values of protein denaturation. The presence of lipid interferes with foaming/whipping; thus, UF WPC must be defatted to function well, while Spherosil QMA WPI is a very good foaming/whipping product. More than 50% of the protein is denatured in Vistec and Spherosil S WPIs; however, Vistec WPI is a good emulsifier, perhaps because β-lactoglobulin when thermally denatured at alkaline pH remains soluble at pH 6. Differences between Vistec and Spherosil S WPI point to the importance of the structure and/or composition of the denatured state in the determination of functionality. Because undenatured forms of whey proteins are soluble under acid condition, they should have applications in acid-type food formulations requiring solubility, such as carbonated fortified beverages.

In some applications, aggregation of the protein is important to texture and rheological properties, for example, in baking or textured food products. Again, however, the importance of the structure of the denatured, associated state must be emphasized. The development of fat mimetics using whey proteins illustrates the importance of control of the extent and type of protein denaturation to achieve a desired functionality in the final state.

15.5.2.4 Lactose

Uses of lactose depend on its low relative sweetness, protein stabilizing properties, crystallization habit, ability to accentuate flavor, nutritional attributes (inclusion of lactose in the diet improves utilization of calcium and other minerals), and ability to engage in Maillard browning [50]. The relative sweetness of lactose is commonly reported as being one-sixth that of sucrose; however, more recent studies indicate that its relative sweetness varies with concentration, ranging from one-half to one-fourth that of sucrose. Since, sugars are often used to increase viscosity and/or mouth feel, or to improve texture, lactose can function in these capacities without being too sweet. Thus, lactose can replace a portion of the sucrose in toppings and icings to improve appearance and stability and to reduce sweetness. Unlike many other food sugars, the addition of lactose does not reduce the solubility of sucrose. Furthermore, as the concentration of lactose is increased, the crystal habits of both sucrose and lactose change. Crystals of both become smaller, thereby yielding a softer, smoother product. Hence, the quality of certain candy and confectionery products can be substantially improved by the addition of lactose.

One of the principal functions of lactose in baked goods is to improve crust color and toasting qualities via the Maillard browning reactions. When added to biscuits, it also increases volume and tenderness and improves texture. Moreover, in baked goods containing yeast, lactose remains available for browning because it is not utilized by the yeast.

In common with other polyols, lactose stabilizes protein structure in solution. Thus, it has been used, for example, to reduce insolubilization of lipovitellin during freeze-drying and to preserve the activity of such enzymes as chymosin during spray-drying.

15.5.2.5 Functionality of Milk Lipids

Milk lipids contribute unique characteristics to the appearance, texture, flavor, and satiability of dairy foods. Consequently, it is difficult to find suitable substitutes for milk fat in nonfat or low-fat foods. Currently, protein- or carbohydrate-based fat mimetics provide some but not all of the functions of the fat replaced. Fat mimetics that possess desirable flavor and satiability are especially difficult to find.

Structures of triacylglycerols in a composite fat are responsible for its melting point, crystallization behavior, and rheological properties. Consequently, alteration in the triacylglycerol composition will affect these properties. Fractionation of milk fat, for example, by progressive crystallization or supercritical fluid technology, can yield lipid fractions with unique functionalities desirable for specific applications in products such as spreads, pastries, or confectionery products. Fatty acid composition of the triacylglycerol can be altered by interesterification in the presence of specific added fatty acids. This reaction, catalyzed by immobilized lipase, and perhaps combined with lipid fractionation, offers an opportunity for modifying milk fat to achieve specific functionalities.

15.6 NUTRITIVE VALUE OF MILK

15.6.1 Nutrient Composition of Milk and Milk Products

Dairy foods make a significant contribution to the total nutrient diet of the U.S. population. For example, Americans obtain one-fourth or more of their protein, calcium, phosphorus, and riboflavin from dairy foods (Figure 15.7). The nutrient composition of milk is shown in Table 15.17. The composition listed, which applies to fresh raw or pasteurized whole milk, does not include vitamin D because this nutrient is usually added to fluid milk at a level of 41 IU/100 g, which is equivalent to about 35% of the RDA (recommended dietary allowance) per 250 mL.

Calculation of nutrient density, that is, the percent of RDA provided in a specified caloric portion or weight of a food product, provides a meaningful method of comparing the nutritive quality of various foods [23,36]. Choosing 2,000 kcal (8372 kJ) as the basis for comparison, which is roughly the average recommendation for adults in the U.S. population, the percentages of the RDA contained

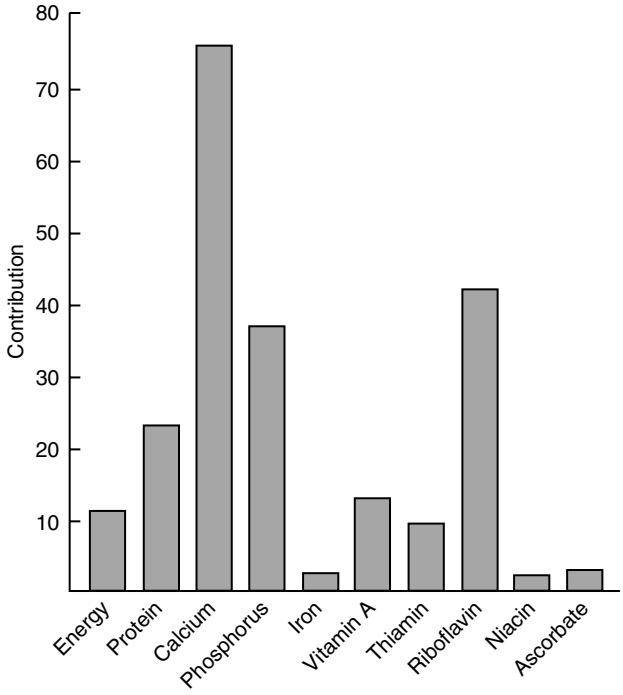

FIGURE 15.7 Contribution of dairy foods to total nutrient consumption in the United States. (From Leveille, G. A. (1979). *J. Dairy Sci.* 62:1665–1672.)

TABLE 15.17
Nutrient Composition of Whole Milk (3.3% Fat)

Nutrient[a]	Amount in 100 g	%RDA[b] in 250 mL
Protein	3.29 g	17.2
Vitamin A	31 RE[c]	8.9
Vitamin C	0.94 mg	4.2
Thiamine	0.038 mg	8.2
Riboflavin	0.162 mg	30.0
Niacin	0.85 NE[d]	13.9
Vitamin B_6	0.042 mg	6.5
Folacin	5 μg	6.4
Vitamin B_{12}	0.357 μg	46.1
Calcium	119 mg	32.0
Phosphorus	93 mg	25.0
Magnesium	13 mg	10.2
Iron	0.05 mg	0.9
Zinc	0.38 mg	6.5

[a] Includes all nutrients for which RDA are available, with the exception of vitamin D, vitamin E, and iodine, for which compositional data were not given in USDA Handbook 8-1.
[b] Average RDA for all males and females above age 11. A 250 mL volume is slightly more than one cup.
[c] RE = Retinol equivalents: 1 μg retinol or 6 μg β-carotene.
[d] NE = Niacin equivalents: 1 mg niacin or 60 mg dietary tryptophan. Only 10% of the NE in milk is in the form of niacin.

Note: Values calculated from the recommended daily allowances (RDA) given by the National Academy of Sciences [55] and the composition given in USDA Handbook No. 8-1 [1].

in one serving of various milk products can be calculated. For example, a 240-mL serving of 1% low-fat milk contains RDA percentage values of 4% for total fat, 7% for saturated fat, 3% for cholesterol, 5% for sodium, and 4% for total carbohydrate. Both 2,000 and 2,500 kcal (10,465 kJ) are used as a basis of nutrient density calculations on food labels (Figure 15.8). Although several nutrients are no longer listed on the label, milk is obviously an excellent source of protein, riboflavin, vitamin B_{12}, calcium, and phosphorus, and a good source of vitamin A, thiamin, niacin equivalents, and magnesium. Removal of fat from milk removes most of the fat-soluble vitamins originally present (A, D, and E), but the nutrient density of other nutrients is significantly increased. In the preparation of cheese, the water-soluble vitamins are significantly lowered due to their partial elimination in whey. Calcium, however, is not reduced in renneted cheeses, as opposed to acid cheeses, because it remains in the clotted casein micelles.

The nutritional quality of a protein is usually a more important consideration than its quantity. Milk proteins correspond very well to human requirements and therefore are regarded as a high quality. The biological value of protein in raw milk is 0.9, based on a value of 1.0 for whole egg protein [58]. Milk proteins are slightly deficient in the sulfur amino acids, methionine, and cysteine, causing the biological value to be slightly less than ideal. Since sulfur amino acids are present in greater amounts in whey proteins than in caseins, the former have a higher biological value (1.0) than the latter (0.8). It is also noteworthy that caseins are readily digestible as a result of their structure.

Whole milk

Nutrition facts
Serving size 1 cup (240 mL)

Amount per serving	
Calories 150	Calories from fat 70
	% Daily value[a]
Total fat 8 g	13%
Saturated fat 5 g	26%
Cholesterol 35 mg	11%
Sodium 120 mg	5%
Total carbohydrate 12 g	4%
Dietary fiber 0 g	0%
Sugars 12 g	
Protein 8 g	
Vitamin A 6% •	Vitamin C 4%
Calcium 30% •	Iron 0%

[a] Percent daily values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2000	2500
Total fat	Less than	65 g	80 g
Sat. fat	Less than	20 g	25 g
Cholesterol	Less than	300 mg	300 mg
Sodium	Less than	2400 mg	2400 mg
Total carbohydrate		300 g	375 g
Dietary fiber		25 g	30 g

Low-fat milk (1%)

Nutrition facts
Serving size 1 cup (240 mL)

Amount per serving	
Calories 110	Calories from fat 20
	% Daily value[a]
Total fat 2.5 g	4%
Saturated fat 1.5 g	7%
Cholesterol 10 mg	3%
Sodium 130 mg	5%
Total carbohydrate 12 g	4%
Dietary fiber 0 g	0%
Sugars 12 g	
Protein 9 g	
Vitamin A 10% •	Vitamin C 4%
Calcium 30% •	Iron 0%

[a] Percent daily values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2000	2500
Total fat	Less than	65 g	80 g
Sat. fat	Less than	20 g	25 g
Cholesterol	Less than	300 mg	300 mg
Sodium	Less than	2400 mg	2400 mg
Total carbohydrate		300 g	375 g
Dietary fiber		25 g	30 g

Cheese (cheddar)

Nutrition facts
Serving size 1 Oz. (28 g)
Servings per container 16

Amount per serving	
Calories 110	Calories from fat 80
	% Daily value[a]
Total fat 9 g	14%
Saturated fat 6 g	30%
Cholesterol 30 mg	10%
Sodium 170 mg	7%
Total carbohydrate 0 g	0%
Dietary fiber 0 g	0%
Sugars 0 g	
Protein 7 g	
Vitamin A 6% •	Vitamin C 0%
Calcium 20% •	Iron 0%

[a] Percent daily values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2000	2500
Total fat	Less than	65 g	80 g
Sat. fat	Less than	20 g	25 g
Cholesterol	Less than	300 mg	300 mg
Sodium	Less than	2400 mg	2400 mg
Total carbohydrate		300 g	375 g
Dietary fiber		25 g	30 g

Yogurt

Nutrition facts
Serving size 1 cup (225 g)

Amount per serving	
Calories 230	Calories from fat 20
	% Daily value[a]
Total fat 2.5 g	4%
Saturated fat 1.5 g	8%
Cholesterol 10 mg	3%
Sodium 130 mg	5%
Total carbohydrate 43 g	4%
Dietary fiber 0 g	0%
Sugars 34 g	
Protein 10 g	
Vitamin A 2% •	Vitamin C 2%
Calcium 35% •	Iron 0%

[a] Percent daily values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2000	2500
Total fat	Less than	65 g	80 g
Sat. fat	Less than	20 g	25 g
Cholesterol	Less than	300 mg	300 mg
Sodium	Less than	2400 mg	2400 mg
Total carbohydrate		300 g	375 g
Dietary fiber		25 g	30 g

FIGURE 15.8 Typical new food labels for several dairy foods.

15.6.2 Lactose Tolerance

A rather high percentage of African and Asian populations produce less intestinal β-galactosidase (lactase) than do Europeans or North Americans. Consequently, lactose maldigestion is encountered more frequently in African and Asian populations than in other populations. Symptoms of lactose maldigestion are diarrhea, bloating, and abdominal cramps [23]. Lactase deficiency, however, is usually one of degree rather than totality [20,23,36]. Since the common lactose intolerance test is conducted by administering a very large dose of 1–2 g lactose/kg body weight, the reported frequency of lactose intolerance is overestimated. Thus, intolerance to a 240-mL glass of milk is infrequently observed among individuals classified as lactose intolerant [20]. Furthermore, in certain cases, milk intolerance is apparently not caused by lactose maldigestion. Nevertheless, a significant percentage of the population benefits from preconsumption hydrolysis of lactose in milk. The means for doing this industrially, using soluble or immobilized microbial lactase, have been developed. Because relatively few persons are intolerant to lactose in low doses, and because those who are can be provided with either lactose-hydrolyzed milk or commercially available lactase in liquid or tablet form, milk products should be regarded as an important part of a varied and balanced diet and as an excellent food for remedying dietary deficiencies on a worldwide basis.

15.6.3 Effect of Processing on Nutritive Value

15.6.3.1 Effect on Proteins

Heating is involved in most types of processing, including pasteurization, sterilization, concentration, and dehydration. Effects of processing can be divided into two categories: (1) those that alter secondary, tertiary, and quaternary structure of proteins and (2) those that alter the primary structure of proteins. The former effects, which lead to protein unfolding, may actually improve the biological value of a protein because peptide bonds become more accessible to digestive enzymes. However, modification of the primary structure may lower digestibility and produce residues that are not biologically available. Heat treatment of milk can cause β-elimination of cystinyl and phosphoseryl residues forming dehydroalanine. This substance readily reacts with lysyl residues to give lysinoalanine cross-links in the protein chain. Lysinoalanine is not biologically available, and the cross-linking lowers digestibility of the protein. Furthermore, because the nutritive value of milk proteins is limited by the low content of sulfur amino acids, such changes are particularly significant. Fortunately, pasteurization or UHT processing does not result in significant formation of lysinoalanyl residues; however, in-can sterilization or boiling does.

Even mild heat treatment initiates Maillard reactions, forming lactulose-lysine and other compounds that reduce the amount of available lysine. Loss of available lysine is not significant during pasteurization (1–2%) or UHT sterilization (2–4%); however, more severe treatments, such as high-temperature evaporative concentration or in-can sterilization, can cause losses of more than 20% [56]. Storage of UHT products for long times at temperatures above 35°C can also significantly reduce available lysine. Because milk proteins contain an abundance of lysine, small losses are not nutritionally significant except in cases in which milk products are used to compensate for lysine-deficient diets.

15.6.3.2 Effect on Vitamins

The fat-soluble vitamins in milk, A (also carotene), D, and E, and the water-soluble vitamins, riboflavin, pantothenic acid, biotin, and nicotinic acid, are quite stable [56]. Accordingly, these vitamins do not sustain any detectable loss during pasteurization or UHT sterilization (Table 15.18). However, thiamine, B_6, B_{12}, folic acid, and ascorbate (vitamin C) are more susceptible to heat and/or oxidative degradation. Vitamins C, B_{12}, and folic acid are particularly susceptible to oxidative degradation during processing and subsequent storage. The first oxidation product of vitamin C

TABLE 15.18
Effects of Processing on Nutrients in Milk

Nutrient	Pasteurization[a]	UHT Sterilization[b]	Spray Drying[c]	Evaporated Milk[d]
Vitamin A	0	0	0	0
Thiamin	10	10	10	40
Riboflavin	0	0	0	0
Nicotinic acid	0	0	0	5
Vitamin B_6	0	10	0	40
Vitamin B_{12}	10	10	30	80
Vitamin C	10–25	25	15	60
Folic acid	10	10	10	25
Pantothenic acid	0	0	0	0
Biotin	0	0	10	10

[a] Heated at 71–73°C for 15 s.
[b] Heated at 130–150°C for 1–4 s.
[c] Pretreated by heating at 80–90°C for 10–15 s, homogenized, and evaporated at reduced pressure. Spray-dried by exposing a fine mist of milk concentrate to air at 90°C for 4–6 s.
[d] Pretreated by heating at 95°C for 10 min, concentrated by evaporation at 50°C under reduced pressure, and sterilized in the can by autoclaving at 115°C for 15 min.

Note: The values listed in the table are the percentage losses resulting from processing.

Source: Adapted from Rolls, B. A. (1982). In *Handbook of Nutritive Value of Processed Food*, vol. I (M. Rechcigl, ed.), CRC Press, Boca Raton, FL, pp. 383–399.

(dehydroascorbate; has vitamin C activity) is very heat sensitive, whereas ascorbate is quite heat stable. Hence, methods that exclude or remove oxygen during processing, and packages that exclude oxygen during storage, serve to protect these vitamins.

In general, pasteurization and UHT sterilization of milk under proper conditions cause much less vitamin loss than those that occur during normal household food preparation. Furthermore, if the product is stored for long periods, the type of packaging and storage conditions are very important. In addition to exclusion of oxygen, exclusion of light is also important, not only to protect against development of off-flavor, but also to prevent loss of riboflavin.

Severe heat treatments, such as in-can sterilization or dehydration, can cause significant losses of many of the vitamins. This is of special concern for vitamin B_{12}, because milk is an important dietary source of this vitamin. Knowledge of the causes of milk degradation and the use of methods to minimize degradation, such as the use of high-temperature-short-time processing and exclusion of oxygen and light during processing and storage, should in the future lead to milk products that are shelf stable, microbiologically safe, and almost unchanged with respect to original nutrients.

15.7 BIOACTIVE PEPTIDES DERIVED FROM MILK PROTEINS

Milk proteins contain peptide sequences that affect various biological functions such as hormone secretion, immune defense, nutrient absorption, transmission of neurological information, and microbial growth [10,11,18]. These peptides are released *in vivo* by proteolytic activity in the stomach and/or the intestines. The peptides could also be produced *in vitro* from specific milk proteins using the appropriate proteinase or they could be produced by chemical synthesis. The peptides thus produced could then be added to foods or other products for specific purposes.

TABLE 15.19
Bioactive Peptides Derived from the Caseins of Milk

Protein	Common Name	Peptide	Biological Activity
α_{S1}-Casein	Isracidin	α_{S1}-Casein(f 1–23)	Antimicrobial
	α_{S1}-Casokinin-5	α_{S1}-Casein(f 23–27)	Antimicrobial and ACE inhibitor
	Caseinophosphopeptide	α_{S1}-Casein(f 59–79)	Calcium binding and transport
	α-Casein exorphin	α_{S1}-Casein(f 90–96)	Opioid agonist
	Casoxin D	α_{S1}-Casein(f 158–164)	Opioid antagonist
α_{S2}-Casein	Casocidin-I	α_{S2}-Casein(f 165–203)	Antimicrobial
β-Casein	β-Casokinin-7	β-Casein(f 177–183)	ACE inhibitor
	β-Casokinin-10	β-Casein(f 193–202)	ACE inhibitor and immunomodulatory
	β-Casomorphin-5	β-Casein(f 60–64)	Opioid agonist
	Morphiceptin	β-Casein(f 60–63 amide)	Opioid agonist
κ-Casein	Casoplatelin	κ-Casein(f 106–116)	Antithrombotic
	Thrombin inhibitory peptide	κ-Casein(f 112–116)	Antithrombotic
	Glycomacropeptide	κ-Casein(f 106–169)	Inhibits platelet aggregation, anticariogenic, and immunomodulatory
	Casoxin C	κ-Casein(f 25–34)	Opioid antagonist

15.7.1 Derived from Caseins (Table 15.19)

A number of peptides (casokinins) derived from α_{S1}- and β-casein exhibit antihypertensive activity because they inhibit angiotensin-converting enzyme (ACE). Because ACE converts angiotensin I to angiotensin II, ACE inhibitors lower blood pressure and aldosterone and activate the depressor action of bradykinin. Two peptides with antimicrobial activity, Isracidin and Casocidin-I, are derived from α_{S1}- and α_{S2}-caseins, respectively. These peptides are active against both Gram (+) and Gram (−) strains. The glycomacropeptide derived from κ-casein during cheesemaking exhibits anticariogenic activity due to its inhibition of the growth of oral streptococci and formation of dental plaque. Thus, it is used in some oral health care products. The phosphopeptide domains of the calcium-sensitive caseins have excellent calcium-binding properties and are resistant to proteolysis. Hence, these caseinophosphopeptides, derived from α_{S1}-, α_{S2}-, and β-caseins, are present in the small intestine where they increase calcium solubility and enhance calcium transport across the intestinal wall; thus increasing calcium absorption and bone calcification.

A number of peptides that have opioid activity have been identified. Several derived from β-casein are known as β-casomorphins because of their morphine-like activity. Although their primary structure differs slightly from typical opioid peptides known as endorphins, these atypical peptides are opioid agonists because their structures fit well in the binding pocket of the opioid receptor. As a result they can modulate physiological activities such as gastrointestinal transient time, antidiarrheal action, amino acid transport, and endocrine activity of the pancreas causing an increase in insulin output. Two peptides, Casoxin D from α_{S1}-casein and Casoxin C from κ-casein, act as opioid antagonists.

Several peptides, principally from κ-casein, display antithrombotic and/or immunomodulating activities. For example, the glycomacropeptide promoted synthesis of IgA and induce proliferation of B-lymphocytes. It also inhibits platelet aggregation thus decreasing thrombosis. Several other peptides derived from the glycomacropeptide region of κ-casein also have antithrombotic activity.

15.7.2 Derived from Whey Proteins (Table 15.20)

A number of antimicrobial peptides derived from β-lactoglobulin and α-lactalbumin have been identified. They appear to be active only against Gram (+) bacteria. The β-lactorphins derived from β-lactoglobulin exhibit ACE inhibitor activity and one is also an opioid agonist.

TABLE 15.20
Bioactive Peptides Derived from the Whey Proteins of Milk

Protein	Common Name	Peptide	Biological Activity
β-Lactoglobulin	None	β-Lactoglobulin(f 15–20)	Antimicrobial
	None	β-Lactoglobulin(f 25–40)	Antimicrobial
	None	β-Lactoglobulin(f 78–83)	Antimicrobial
	None	β-Lactoglobulin(f 92–100)	Antimicrobial
	β-Lactorphin	β-Lactoglobulin(f 142–148)	ACE inhibitor
	β-Lactorphin (amide)	β-Lactoglobulin(f 102–105)	Opioid agonist, ACE inhibitor
α-Lactalbumin	None	α-Lactalbumin(f 17–31S–S109–114)	Antimicrobial
	None	α-Lactalbumin(f 61–68S–S75–80)	Antimicrobial
Lactoferrin	Lactoferricin B	Lactoferrin(f 17–41)	Antimicrobial

The best known and well-characterized antimicrobial peptide is lactoferricin B derived from lactoferrin. Studies have shown that consumption of infant formulas or adult drinks supplemented with lactoferrin will produce relatively high levels of the peptide in the gastrointestinal tract that could have beneficial effects. Lactoferricin B displays bacteriocidal activity against pathogens such as *Staphylococcus aureus* and *Escherichia coli* as effectively as antibiotics. Additional biological activities are also associated with lactoferrin B. The peptide induced apoptosis in a leukemic cell line that was mediated by intracellular reactive oxygen species and activated by endonucleases. Antiviral, immunoregulatory, and anti-inflammatory properties have also been ascribed to the peptide.

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16 Physiology and Chemistry of Edible Muscle Tissues

Gale Strasburg, Youling L. Xiong, and Wen Chiang

CONTENTS

16.1 INTRODUCTION

Archeological evidence indicates that humans have utilized animal products, including meat, as sources of food for thousands of years. This fact was dramatically illustrated following the discovery by mountain hikers in 1991 of Eis Mann (Ice Man), the frozen remains of a man found in a glacier high in the Italian Alps. Ötzi, as he affectionately came to be known by the area residents, had apparently died approximately 5100–5300 years ago. Various lines of evidence suggest that he was a hunter who may have died of arrow wounds as a result of a rivalry with another hunter or hunting groups [1]. His body was so well preserved by the glacial ice that it was possible for scientists to use recombinant DNA methodology to analyze the contents of his gastrointestinal tract in order to determine what he had eaten in his last two meals. The penultimate meal prior to his death consisted of meat from an ibex (a type of wild goat once found in the Alps) as well as cereal grains and other types of plant food. His final meal included red deer meat and possibly cereal grains.

Over the course of history, some cultures have eaten meat as a matter of choice, while others have done so as a matter of necessity. Likewise, religious and cultural factors have had considerable influence in defining meat that is considered acceptable for consumption vs. meat that is considered unacceptable. For example, some religious groups prescribe avoidance of pork whereas others mandate avoidance of beef. In the United States, eating horse meat is culturally considered taboo, while it is acceptable in other countries. Still other individuals avoid meat completely for ethical reasons or presumptive health concerns. Nevertheless, it is also evident that as economies of nations grow, particularly in developing countries, there is a parallel growth in demand for meat products.

The term "meat" in colloquial use sometimes connotes red muscle food (beef, pork, lamb), while poultry and fish muscle foods are in classes by themselves. In this chapter, we utilize the term "meat" more broadly to refer to skeletal muscle tissue from a mammal, bird, reptile, amphibian, or fish that has undergone a specific series of transformative biochemical reactions following death of the animal. This term specifically excludes other organ meats such as liver, thymus, and kidney, but does include heart and tongue as unique muscle tissues. The purpose of this chapter is to provide the reader with an essential understanding of muscle physiology and biochemistry, which in turn lays the foundation for understanding the biochemical events in conversion of muscle to meat and of subsequent processing factors related to the functional properties of meat components.

16.2 NUTRITIVE VALUE

The positive sensory appeal of fresh and processed meat products, as well as the feeling of satiety following consumption of a meal including meat, have combined to make muscle food products staples of human diets around the world. The high nutrient density of meat, that is, the concentrations of nutrients per kilocalorie, the variety of nutrients present in the tissues, and the excellent bioavailability of nutrients combine to make meat a significant source of nutrition for consumers.

The composition of meat is quite variable. Species, breed, sex, age, nutritional status, and activity level of the animal are major factors affecting the gross composition of meat [2]. Moreover, even for a given animal, the anatomical location of the retail cut, postslaughter processing, storage, and of course, cooking, contribute significantly to the variability of meat composition. It is beyond the scope of this chapter to examine these factors beyond a few generalizations discussed below. Detailed information on composition of meat products by species, retail cut, degree of trim, raw vs. cooked product, and so forth is available from the USDA and is periodically updated [3]. However, even these values must be regarded as approximations, given the many sources of variation.

The proximate composition of separable lean tissue (skeletal muscle trimmed of external fat) is somewhat variable, but in general, water accounts for about 70% of the weight of fresh, lean muscle (Table 16.1). The water is largely trapped within or between the muscle cells, with lesser amounts bound in varying degrees to proteins. Variation in water content is generally offset by changes in

TABLE 16.1
Proximate Composition of Meat from Various Sources[a]

	Red Meat			Poultry		Fish	
	Beef	**Pork**	**Lamb**	**Chicken**	**Turkey**	**Cod**	**Tuna**
Water	70.62	72.34	73.42	74.76	74.12	81.22	68.09
Protein	20.78	21.07	20.29	23.09	24.60	17.81	23.33
Lipid	6.16	5.88	5.25	1.24	0.65	0.67	4.90
Ash	1.02	1.04	1.06	1.02	1.02	1.16	1.18

[a] Percent by weight of edible portion.

Source: Compiled from U.S. Department of Agriculture, Agricultural Research Service (2005). *Composition of Foods Raw, Processed, Prepared.* USDA National Nutrient Database for Standard Reference, Release 18. Nutrient Date Laboratory Home Page (http://www.nal.usda.gov/fnic/foodcomp/).

TABLE 16.2
Lipid Content of Various Meats

		Content[a]		
Species	**Muscle or Type**	**Lipid (%)**	**Neutral Lipids (%)**	**Phospholipids (%)**
Chicken	White	1.0	52	48
	Red	2.5	79	21
Turkey	White	1.0	29	71
	Red	3.5	74	26
Fish (sucker)	White	1.5	76	2
	Red	6.2	93	7
Beef	Lattisimus dorsi	2.6	78	22
		7.7	92	8
		12.7	95	5
Pork	Lattisimus dorsi	4.6	79	21
	Psoas major	3.1	63	37
Lamb	Lattissimus dorsi	5.7	83	10
	Semitendinosus	3.8	79	17

[a] Percentage of gross muscle composition.

Source: Allen, C.E. and E.A. Foegeding (1981). *Food Technol*. 35:253–257.

lipid composition, while protein composition ranges from 18% to 23%, and ash or mineral content is approximately 1–1.2% (Table 16.1).

The lipid content and composition are the most variable of the four primary components of meat. Since the lipid fractions associated with muscle tissue and adipose tissue vary in quantity and composition, the amount of adipose tissue present in a meat product profoundly affects the proximate composition of the product [4]. Moreover, as the adipose tissue content of meat decreases, the percentage of phospholipid contribution to the total increases (Table 16.2). Most of the lipid in meat consists of neutral triacylglycerols, lesser amounts of phospholipids that comprise cellular membranes, and a small amount of cholesterol found primarily in the muscle plasma membrane and nervous tissue. Fatty acids in the neutral fat fraction tend to be more highly saturated than those of the

TABLE 16.3
Fatty Acid Composition of Meat from Various Sources[a]

	Red Meat			Poultry		Fish	
	Beef[b]	Pork[c]	Lamb[d]	Chicken[e]	Turkey[f]	Cod[g]	Tuna[h]
Total saturated	37.66	34.52	35.81	26.61	32.31	19.55	25.65
14:0	2.76	1.19	2.67	0.81	0.00	1.34	2.84
16:0	22.73	21.94	19.43	16.94	13.85	13.58	16.53
18:0	11.85	10.88	11.81	8.06	9.23	4.48	6.27
Total monounsaturated	42.37	45.24	40.19	24.19	16.92	14.03	32.65
16:1	3.73	3.23	3.05	2.42	1.54	2.39	3.31
18:1	38.31	41.16	36.38	20.16	13.85	9.10	18.86
Total polyunsaturated	3.90	10.71	9.14	22.58	26.15	34.48	29.24
18:2	3.08	8.67	6.86	13.71	16.92	0.75	1.08
18:3	0.32	0.34	1.33	0.81	0.00	0.15	0.00
20:4	0.49	1.19	0.95	3.23	4.62	3.28	0.88

[a] Percent of total fat in lean compiled from all retail cuts for red meat and in breast for poultry meat and in whole fish. Calculated based on the information compiled from U.S. Department of Agriculture, Agricultural Research Service (2005). *Composition of Foods Raw, Processed, Prepared*. USDA National Nutrient Database for Standard Reference, Release 18. Nutrient Date Laboratory Home Page (http://www.nal.usda.gov/fnic/foodcomp/).

[b] Beef, composite of trimmed retail cuts, separable lean only, trimmed to 1/4" fat, all grades, raw.

[c] Pork, fresh, composite of trimmed retail cuts (leg, loin, shoulder), separable lean only, raw.

[d] Lamb, domestic, composite of trimmed retail cut, separable lean only, trimmed to 1/4" fat, choice, raw.

[e] Chicken, broilers or fryers, breast, meat only, raw.

[f] Turkey, fryer-roasters, breast, meat only, raw.

[g] Cod, Atlantic, raw.

[h] Tuna, bluefin, raw.

phospholipid fraction, which is not surprising from a functional standpoint. The necessity of a fluid cell membrane at physiological temperatures requires that phospholipids have a higher percentage of unsaturated fatty acids, while a high fraction of saturated fatty acids with their higher melting points is required to maintain the integrity of adipose tissue. Despite the smaller contribution of phospholipid fraction to the total lipid composition, the polyunsaturated nature of the phospholipids together with the high surface to volume ratio makes this lipid highly susceptible to oxidative reactions that contribute to deterioration of flavor and color of meat [5].

Lipid composition varies by species, with the highest levels of polyunsaturated fatty acids found in fish, and lowest amounts in beef and mutton (Table 16.3). Lipid composition also varies from muscle to muscle within a species, particularly when comparing muscles in which most of the fibers rely on oxidative metabolism (red muscles) to muscles that generally rely on glycolytic metabolism (white muscles).

The protein content of meat is typically derived from analysis of total nitrogen content of the product, multiplied by 6.25, a factor based on the average nitrogen content of meat protein. However, this approach overestimates the amount of protein, because as much of 10% of muscle nitrogen comes from nonprotein sources including amino acids, peptides, creatine, nucleic acids, and other nitrogen-containing molecules.

Meat is an excellent source of dietary protein because the amino acid composition closely parallels human dietary amino acid requirements. The high quality together with the relative abundance of

TABLE 16.4
Mineral and Vitamin Composition of Meat from Various Sources[a]

	Red Meat			Poultry		Fish		DRI[b]
	Beef	**Pork**	**Lamb**	**Chicken**	**Turkey**	**Cod**	**Tuna**	**RDA or AI***
Minerals								
Potassium	356	380	280	255	293	413	252	4,700/4,700*
Phosphorus	199	211	189	196	206	203	254	700/700
Sodium	63	57	66	65	49	54	39.0	1,500/1,500*
Magnesium	22	23	26	28	28	32	50.0	420/320[c]
Calcium	6	16	10	11	10	16	8.0	1,000/1,000*
Zinc	4.33	2.08	4.06	0.8	1.24	0.45	0.60	11/8
Iron	2.13	0.91	1.77	0.72	1.17	0.38	1.02	8/18
Vitamins								
Thiamin	110	966	130	70	41	76	241	1,200/1,100
Riboflavin	180	273	230	92	118	65	251	1,300/1,100
Niacin	3,590	4,829	6,000	11,194	6,255	2,063	8,654	16,000/14,000
Pantothenic Acid	360	788	700	819	717	153	1,054	5,000/5,000*
B_6	440	500	160	550	580	245	455	1,300/1,300
Folate	7.0	5.0	23.0	4.0	8.0	7	2.0	400/400
B_{12}	3.25	0.67	2.62	0.38	0.47	0.91	9.43	2.4/2.4

[a] Values are expressed as mg/100 g and μg/100 g for minerals and vitamins, respectively. *Source:* Compiled from U.S. Department of Agriculture, Agricultural Research Service (2005). *Composition of Foods Raw, Processed, Prepared*. USDA National Nutrient Database for Standard Reference, Release 18. Nutrient Date Laboratory Home Page (http://www.nal.usda.gov/fnic/foodcomp/).

[b] DRI (Dietary Reference Intakes) values are expressed as RDA (Recommended Dietary Allowances) or AI* (Adequate Intake) for male/female adults (age 19–50). *Source:* Compiled from Food and Nutrition Board, Institute of Medicine, National Academy of Sciences. (1997). Dietary Reference Intakes for calcium, phosphorus, magnesium, vitamin D and floride; Food and Nutrition Board, Institute of Medicine, National Academy of Sciences. (1998). Dietary Reference Intakes for thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin, and choline; Food and Nutrition Board, Institute of Medicine, National Academy of Sciences. (2000). Dietary Reference Intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc; and Food and Nutrition Board, Institute of Medicine, National Academy of Sciences. (2004). Dietary Reference Intakes for water, potassium, sodium, chloride, and sulfate.

[c] Age 31–50.

protein in meat means that a single 85-g serving of meat may provide 50–100% of the daily protein intake recommended for maintenance of growth and health [6]. Moreover, the complete amino acid composition of meat enables complementation of other dietary sources of protein. For example, even a small amount of meat added to a cereal- or legume-based diet, which would be deficient in lysine and sulfur amino acids, respectively, dramatically improves the nutritional value of the plant-derived protein.

Muscle tissue is an excellent source of many water-soluble vitamins including thiamin, riboflavin, niacin, B_6, and B_{12} (Table 16.4). However, as with other nutrients, the vitamin content is strongly influenced by species, age, sex, and nutritional status of the animal. Most noteworthy are the very high levels of thiamin and the low levels of B_{12} in pork compared with beef and lamb. Vitamins C, D, E, and K tend to be low in all muscle foods. However, studies have indicated that levels of vitamin E in meat can be significantly elevated through increased dietary supplementation. Because vitamin E functions as an antioxidant, its presence at elevated levels may have a significant beneficial effect in stabilizing meat color, reducing lipid oxidation, and enhancing human health [7].

Red meats are particularly good sources of iron because of their high myoglobin content; however, even white muscle of poultry and fish can be significant sources of iron (Table 16.4). Moreover, the heme form of the iron imparts a high degree of bioavailability compared with most inorganic sources of iron. Potassium, phosphorus, and magnesium are relatively abundant in meat. Calcium, despite its importance in regulating muscle contraction, is present in muscle at very low levels relative to dietary requirements. In mechanically separated meats, calcium may be present at higher levels because of the presence of small amounts of microscopic bone fragments present in the final product [8].

Carbohydrates make only a small contribution to the composition of fresh meat (<1%). The major source of carbohydrate in muscle is glycogen, with minor amounts of monosaccharides and glycolytic metabolites. During conversion of muscle to meat, glycogen is largely converted to lactate by anaerobic glycolysis, thus making lactate the primary carbohydrate in meat [9].

16.3 STRUCTURE AND FUNCTION OF MUSCLE

16.3.1 Structure of Skeletal Muscle

Individual skeletal muscles vary greatly in size and morphology. In general, they consist of a parallel arrangement of elongated, multinucleated cells called myofibers or muscle fibers. Individual myofibers range from 10 to 100 μM in width, and from a few millimeters to several centimeters long, sometimes spanning the entire length of a muscle. Myofibers are arranged in hierarchical fashion with associated blood, circulatory, nerve, and blood tissues forming the whole muscle organ (Figure 16.1). Each myofiber is encased in a layer of connective tissue called the endomysium. Groups of myofibers are organized into primary and secondary bundles or fascicles that are segregated by another layer of connective tissue called the perimysium. A final layer of heavy connective tissue sheaths, the epimysium, surrounds the whole muscle. These sheaths merge with the connective tissue tendons to link the muscle to the bones. The molecular and structural properties of connective tissue are described in Section 16.3.4.1.

Muscle is infiltrated by a complex system of nerves involved in the regulation of muscle contraction and maintenance of muscle tone, as well as a vascular system through which blood provides oxygen and nutrients while removing metabolic end products (Figure 16.1). The perimysium and endomysium combine to provide the necessary framework for maintaining the structural integrity of these tissues within the muscle at rest, and more importantly during the mechanical stress of contraction. Adipose tissue may also be found embedded in the perimysial layer and is visible in red meats as white flecks of fat (marbling) in contrast to the red background of myofibers. The abundance of marbling is often used as an indicator in visual appraisal of meat quality [10].

The unique structure of muscle cells enables translation of electrochemical impulses, triggered by neural stimulation, into increased intracellular calcium concentrations that in turn trigger muscle contraction. Like all cells, a myofiber is bounded by a plasma membrane, referred to as the sarcolemma (SL) in a muscle (Figure 16.2). However, the skeletal muscle SL is distinguished from the plasmalemma of other cells by periodic invaginations of the membrane into the interior of the muscle cell, much like fingers poking into the skin of a balloon. These inward extensions of the SL, referred to as transverse tubules or T-tubules, transmit the action potential or depolarization signal for contraction from the neuromuscular junction to the interior of the myofiber. The T-tubules are in physical contact at periodic intervals with an extensive, highly developed intracellular membrane network called the sarcoplasmic reticulum (SR), which is the muscle equivalent of the endoplasmic reticulum (Figure 16.2). The SR network encircles the contractile organelles (myofibrils) and functions as a reservoir of calcium ions that serve as the trigger for muscle contraction. Numerous proteins embedded in the SR are responsible for specific functions related to calcium regulation. Some proteins in the interior (lumen) of the SR bind calcium ions while the muscle is at rest [11]. Other proteins form channels that open in response to the depolarization signal, thereby allowing diffusion of calcium ions from within the SR to the sarcoplasm; this process triggers muscle contraction [12]. Another SR

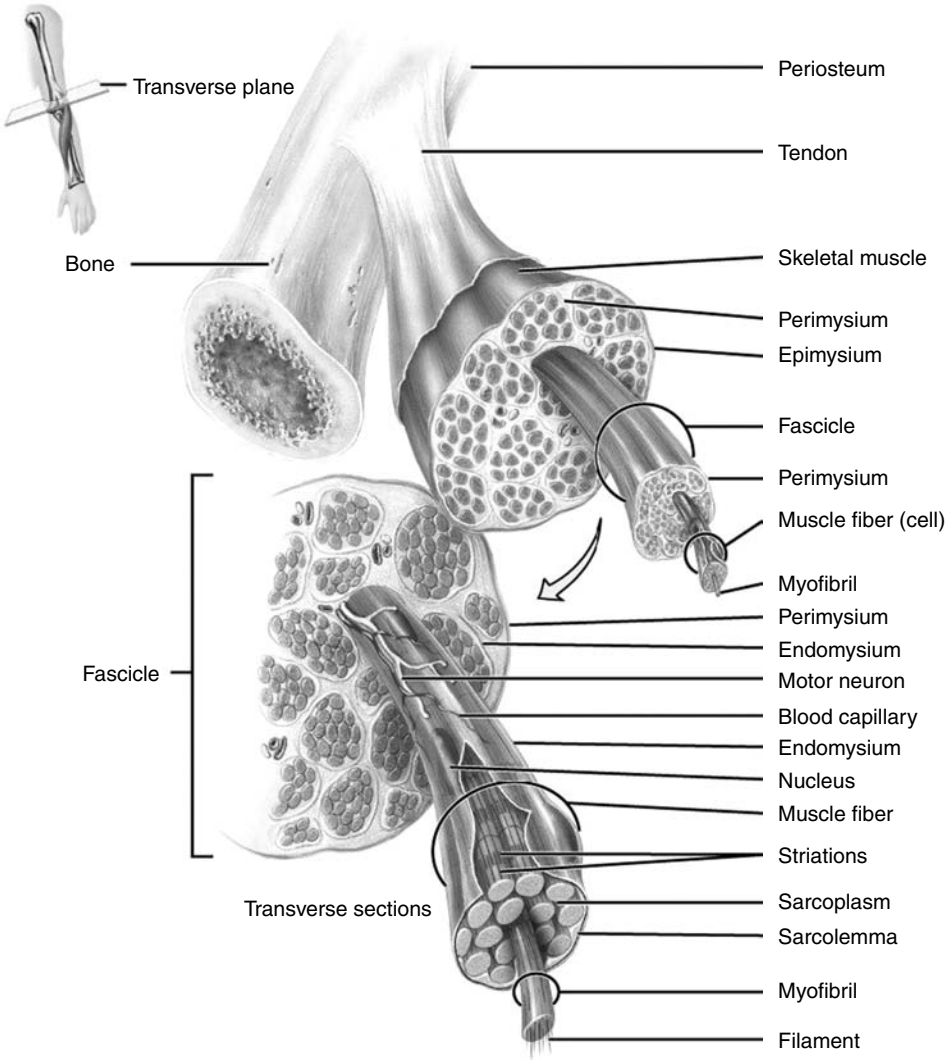


FIGURE 16.1 Diagrammatic representation of the structural organization of muscle from subcellular myofibrils to whole organ. Individual muscle cells (fibers) are surrounded by a layer of connective tissue (endomysium), which, in turn, are organized into bundles (fascicles), separated by another layer of connective tissue called the perimysium. Blood vessels and nerves penetrate the perimysium as supporting tissues for muscle function. (Reprinted from Tortora, G.J. and B. Derrickson (2006). *Principles of Anatomy and Physiology*. John Wiley and Sons, Inc., Hoboken, NJ.)

protein pumps calcium back into the lumen of SR during relaxation. These proteins are discussed in greater detail in Section 16.3.4.6.

Muscle cells possess other organelles typical of all cells. Because of the developmental path by which skeletal muscle cells develop, myofibers are typically multinucleated (Figure 16.2). The nuclei are usually dispersed to the periphery of the cell, and are typically found immediately beneath the SL. Mitochondria serve as energy transducers for the myofiber and are found throughout the cell in close association with myofibrils. Lysosomes serve as a major reservoir for a family of proteolytic enzymes known as cathepsins, which play a catabolic function in protein turnover.

The sarcoplasm (cytoplasm) of muscle may contain glycogen particles and lipid droplets, the quantity of which depend on the type of muscle fiber (oxidative or nonoxidative), and the nutritional

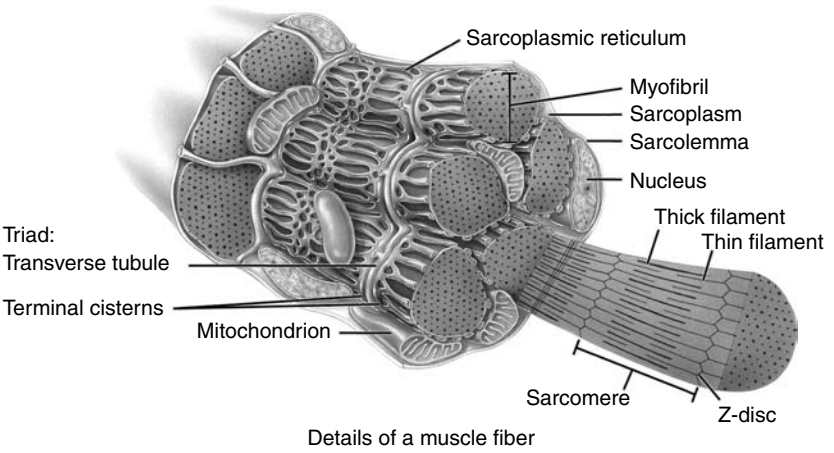


FIGURE 16.2 Schematic representation of the structural organization of a muscle fiber. Multinucleated muscle fibers are encased by the sarcolemma. Invaginations of the sarcolemma into the center of the muscle fiber form structures called transverse tubules (T-tubules). Each T-tubule is connected with two terminal cisternae of the SR, forming a structure called a triad. The SR is an organelle that envelopes the myofibrils, storing calcium ions when muscle is at rest, and releasing calcium ions to the sarcoplasm during muscle contraction. (Reprinted from Tortora, G.J. and B. Derrickson (2006). *Principles of Anatomy and Physiology*. John Wiley and Sons, Inc., Hoboken, NJ.)

and exercise/resting state of the organism. The oxygen-storage protein myoglobin is found to varying degrees within the sarcoplasm, as are various enzymes, metabolic intermediates, and other compounds such as nucleotides, amino acids, and so forth.

Muscle contraction is effected through the action of specialized proteins that are organized into parallel, interdigitating thin and thick filaments (myofilaments) that comprise 80–90% of the volume of the myofiber. Myofilaments are grouped into myofibrils that function in coordinated fashion as the contractile organelles of a muscle cell. The high degree of structural organization of the myofibrils is evident when thin longitudinal sections of skeletal muscle are viewed with a microscope. One sees a pattern of alternating light and dark bands that result from longitudinal repetition of the fundamental structural unit of muscle contraction known as the sarcomere (Figure 16.3a). When viewed with polarized light, the dark bands are anisotropic and are thus referred to as "A-bands." The lighter bands, "I-bands," are isotropic in polarized light. The boundaries of the sarcomere are defined by structures known as Z-discs, which are narrow, dark, electron-dense bands of proteins in the center of the I-band. The term Z-disc is derived from the German *zwischen* meaning "between," indicating its position at the center of the I-band. The matrix of proteins that constitutes the Z-disc serves as the anchoring structure for the proteins of the thin filaments that emanate from both sides of the Z-disc.

The sarcomere consists of alternately placed thin and thick filaments. The I-band consists of thin filaments, while the A-band consists of overlapping thin and thick filaments. The center of the A-band is slightly less dense than the distal regions and therefore appears brighter because this zone consists only of thick filaments with no overlapping thin filaments. This band is called the H-zone, derived from the German *helle* meaning bright. At the center of the H-zone is a dark zone analogous to the Z-disc. This structure, called the M-line, consists of proteins that maintain the structural arrangement of the thick filament proteins and serve as an anchoring point for the protein titin that spans from the M-line to the Z-disc (see Section 16.3.4.5).

In 1954, Huxley and Hanson proposed a theory of muscle contraction, called the sliding filament theory, which has survived largely intact to the present era [13]. The theory is based on the observation that lengths of both thin and thick filaments remain constant, independent of whether the muscle is stretched, contracting, or in the resting state. In contrast, sarcomere lengths, defined as the distance between adjacent Z-discs, vary depending on the state of contraction or stretch force applied to

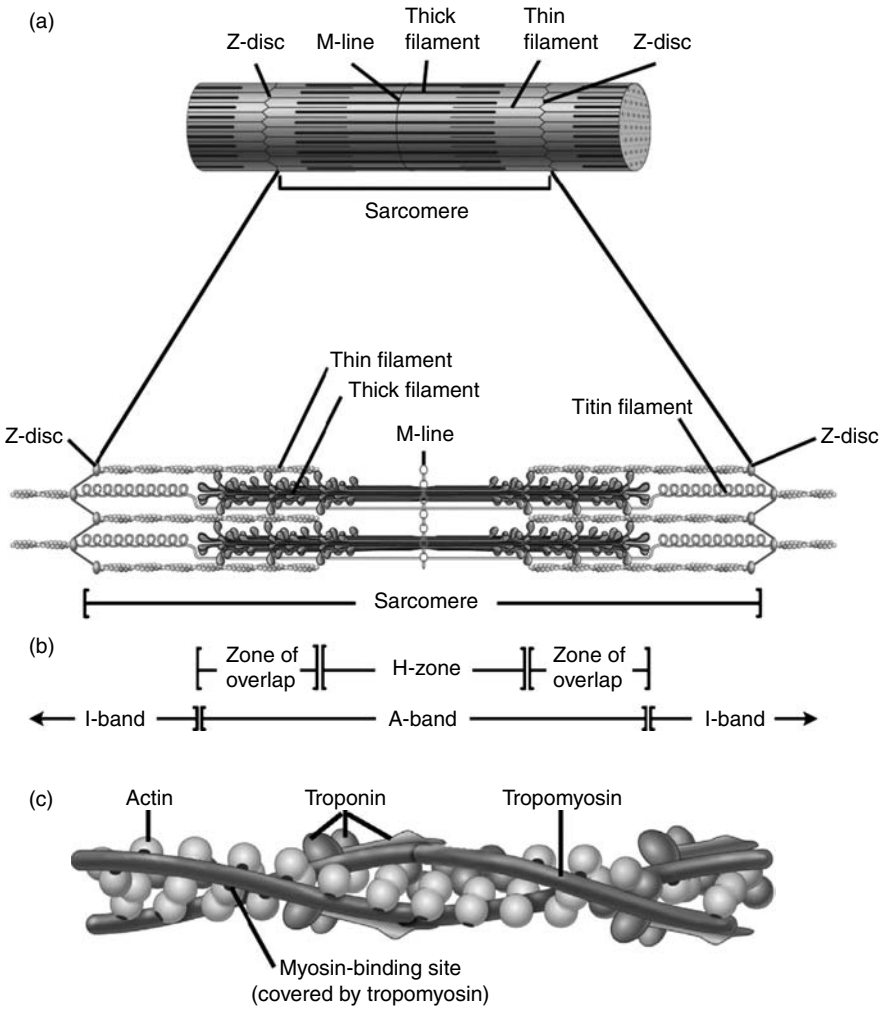


FIGURE 16.3 Structural arrangement of the sarcomere. (a) The sarcomeric unit of a myofibril begins at one Z-disc and extends to the next Z-disc. At the center of each sarcomere is an arrangement of proteins that form the M-line. (b) The major components of the sarcomere are thin filaments, which are anchored at the Z-disc, thick filaments in the central region of the sarcomere that partially overlap with the thin filaments, and titin filaments that span from the Z-disk to the M-line. (c) Actin monomers polymerize to form a double-stranded coiled coil that constitutes the backbone of the thin filament. Tropomyosin polymerizes in "head-to-tail" fashion and lies near the groove of the actin double helix, covering myosin-binding sites on the actin backbone. One tropomyosin molecule spans seven actin monomers. One troponin molecule (consisting of three subunits) binds to one tropomyosin molecule via the asymmetrically shaped troponin-T subunit. (Reprinted from Tortora, G.J. and B. Derrickson (2006). *Principles of Anatomy and Physiology*. John Wiley and Sons, Inc., Hoboken, NJ.)

the fiber. In addition, electron micrographs of muscle cross sections indicated that the thin and thick filaments interdigitate in such a way that a thick filament is surrounded by an array of six thin filaments. Huxley and Hanson proposed that when contraction takes place, the thin and thick filaments slide past each other such that the thin filaments at opposite ends of a sarcomere move toward each other. This results in shortening of the sarcomere length (Figure 16.4). Conversely, stretching results from an increase in Z-disc separation, again accomplished by sliding of thin filaments of a sarcomere away from each other as they move along the A-band. The extent to which thin and thick filaments overlap has great practical significance with respect to meat tenderness. As we shall see

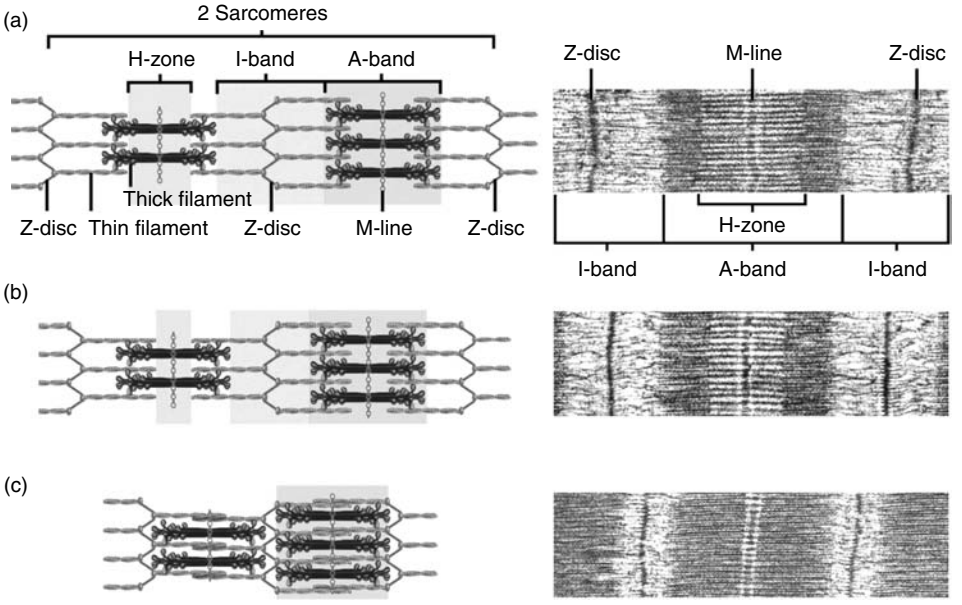


FIGURE 16.4 Illustration of the sliding filament theory: (a) Relaxed; (b) Partially contracted and (c) Maximally contracted. Thin filaments and thick filaments interdigitate allowing for sliding of filaments past each other. Note the shortening of the distance between Z-discs as the muscle undergoes contraction, as well as the increasing overlap of thin and thick filaments as muscle shortens. (Reprinted from Tortora, G.J. and B. Derrickson (2006). *Principles of Anatomy and Physiology*. John Wiley and Sons, Inc., Hoboken, NJ.)

later (Section 16.5.3), there is generally a negative correlation between sarcomere length and meat toughness. When muscle is maximally contracted, the sarcomeres are at their shortest, and the high degree of overlap between filaments together with the large number of rigor bonds formed between the two types of filaments result in increased toughness. The molecular details of how the sliding of filaments results in muscle contraction will be discussed in Section 16.3.5.

16.3.2 Structure of Cardiac Muscle

Heart muscle may be used as a food directly, or more commonly may be minced and incorporated into processed meat products such as sausages. Like skeletal muscle, cardiac muscle is striated, which suggests an arrangement of the contractile proteins of cardiac muscle which is similar to that of skeletal muscle. From an anatomical point of view, the fiber arrangement is somewhat less regular than skeletal muscle fibers, and in contrast to the multinucleated skeletal muscle fibers, cardiac muscle typically has only 1–2 nuclei that are centrally located. Although the proteins comprising the cardiac contractile machinery are the same as skeletal, the isoforms are often specific to cardiac muscle. The differences in amino acid sequence between the skeletal and cardiac isoforms impart differences in protein functionality between proteins from these tissues. In addition, the signaling mechanism for excitation of cardiac muscle fibers and the mechanism of calcium release differ from that of skeletal muscle. All of these factors have important implications with respect to postmortem metabolism of cardiac muscle and utilization of cardiac muscle as a food. A detailed comparison is beyond the scope of this chapter. The reader is advised to consult other references for more details on cardiac muscle biology [14,15].

16.3.3 Structure of Smooth Muscle

Some smooth muscles (e.g., gizzard, stomach, and intestine) are consumed as specialized organ foods. Unlike skeletal and cardiac muscles, smooth muscles do not exhibit the pattern of striation

when longitudinal sections are viewed under the microscope; hence, the term "smooth muscle." This results from a relatively unstructured arrangement of contractile proteins within smooth muscle. Many of the same proteins are involved in smooth muscle contraction as in skeletal muscle, but some are notably absent, and there exist multiple mechanisms of regulation of muscle contraction among different smooth muscles as well as among species. As with cardiac muscle, even when the same proteins are present (e.g., myosin, actin, tropomyosin), the isoforms expressed are tissue-specific and differ sufficiently from the skeletal isoforms such that their functional properties in processed meat products may differ substantially from that of skeletal muscle. The reader is referred to other references for more details on the physiology of smooth muscle [16].

16.3.4 Proteins of the Muscle Tissue

Proteins in skeletal muscle have been categorized according to solubility or biological function. Categories related to biological function generally refer to a protein's contribution to muscle structure, contraction, and metabolism, among others. The solubility category is classically based on differential solubilization of muscle proteins at varying salt concentrations that yields three primary classes of proteins. These classes generally correlate with cellular localization and are identified as (1) sarcoplasmic proteins, (2) myofibrillar proteins, and (3) stromal proteins.

As the name implies, sarcoplasmic proteins comprise proteins found in the sarcoplasm of the myofiber including the glycolytic enzymes, myoglobin, and other enzymes involved in metabolism. These proteins are sometimes termed "water-soluble" proteins because they can be dissolved at low ionic strength ($>$0.3 mM). This fraction constitutes about 30% of the total muscle protein content [17].

The myofibrillar class of proteins constitutes the largest fraction (50–60%) of muscle protein. These proteins require high salt concentrations (e.g., $>$0.3 M NaCl) for solubilization; thus, they are sometimes referred to as the "salt-soluble fraction" of muscle proteins. In muscle tissue, the physiological salt concentration is approximately 0.15 M. This concentration is sufficiently low to prevent these proteins from dissolving in the sarcoplasm, thereby maintaining the complex quaternary structure of the myofilaments.

Myosin and actin, the primary constituents of the thick and thin filaments, respectively, comprise about 65% of the total myofibrillar protein content and about 40% of the total muscle protein content [18] (Figure 16.3a). On the basis of abundance, the chemical behavior of these two proteins in salt solutions accounts for the solubility properties of this group as well as for the development of processed meat products. It must be noted that although the myofibrillar proteins are generally equated with high salt solubility, this is a broad generalization. For example, in some cases complete solubilization of cod myofibrillar proteins has been observed at very low ionic strength ($<$0.0002) [19]. Other myofibrillar proteins such as the troponin complex, when purified, are also soluble at very low ionic strength.

The quality characteristics of fresh meat products are also highly dependent on the abundance and composition of the stromal proteins, which constitute 10–20% of total muscle protein content. The content of stromal proteins varies with species, age, and muscle [20]. These proteins are generally insoluble under the usual conditions of extraction: near-neutral pH, low-salt or high-salt concentrations, and cold temperatures. Collagen, the most abundant protein in the body, is the dominant protein in the stromal fraction. The stromal proteins form the connective tissue layers described previously that strengthen and protect muscles; thus, there may be some correlation of collagen quantity and quality with meat toughness [21]. In addition, collagen molecules are covalently cross-linked (see below), and the number of cross-links increases with age of the animal [22]. The significance of collagen to meat quality is highlighted by the fact that various processing and cookery methods for meat are designed to disrupt and partially solubilize collagen fibers, thereby enhancing meat tenderness [23].

16.3.4.1 Connective Tissue Proteins and Matrix

As noted above, connective tissues permeate the muscle in the form of epimysium, perimysium, and endomysium, and these structures give rise to the strong matrix necessary to support muscle function while maintaining a degree of elasticity [24]. Connective tissue consists of a variety of cell types, including fibroblasts that synthesize collagen, macrophages, lymphoid cells, mast cells, and eosinophils. There is also an extracellular matrix that is composed of fibrous and non-fibrous proteins including members of the collagen family, elastin, and the proteoglycans. Until rather recently, the primary role of extracellular matrix was thought to be maintenance of the structural integrity of the muscle. Since the mid-1990s, it has become apparent that the extracellular matrix plays key roles in regulating the behavior of surrounding cells [25]. The extracellular matrix communicates information to cells via integrin receptors embedded in plasma membranes (Figure 16.5). Integrins serve as linkers between the intracellular cytoskeletal network and the extracellular matrix [26]. Through the integrins, the extracellular matrix regulates and modulates various activities including gene expression, migration, adhesion, proliferation, and differentiation. Differentiation of skeletal muscle is absolutely dependent on proteoglycan synthesis [27], and the types of proteoglycans expressed change as development proceeds [28]. Moreover, it has been suggested that reduced expression level of extracellular matrix proteins [29] as well as postmortem degradation of integrins may contribute to the excessive drip loss associated with poor meat quality [30].

The dominant protein of connective tissue is collagen. The term "collagen" actually refers to a family of at least 27 different protein isoforms found in connective tissues throughout the body including bone, tendon, cartilage, blood vessels, skin, teeth, as well as muscle [31]. Collagen contributes significantly to toughness of mammalian muscle, and in semipure form, it is an important functional ingredient in various food products such as gelatin. Skeletal muscles from small animals and fish have fewer requirements for weight-bearing strength, and tend to be composed of lesser

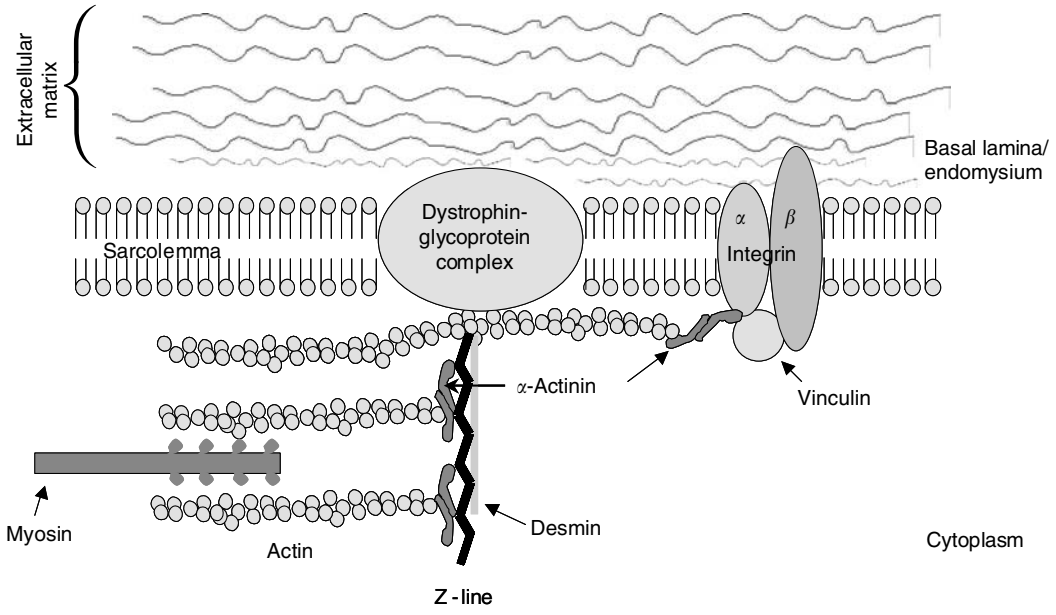


FIGURE 16.5 Diagrammatic representation of the localization and the interaction of extracellular matrix proteins with the intracellular cytoskeletal protein network. Extracellular matrix proteins connect to the intracellular cytoskeleton via the integrin complex embedded in the sarcolemma. (Modified from Lewis, M.P., J.R.A. Machell, N.P. Hunt, A.C.M. Sinanan, and H.L. Tippett (2001). *Eur J Oral Sci* 109:209–221.)

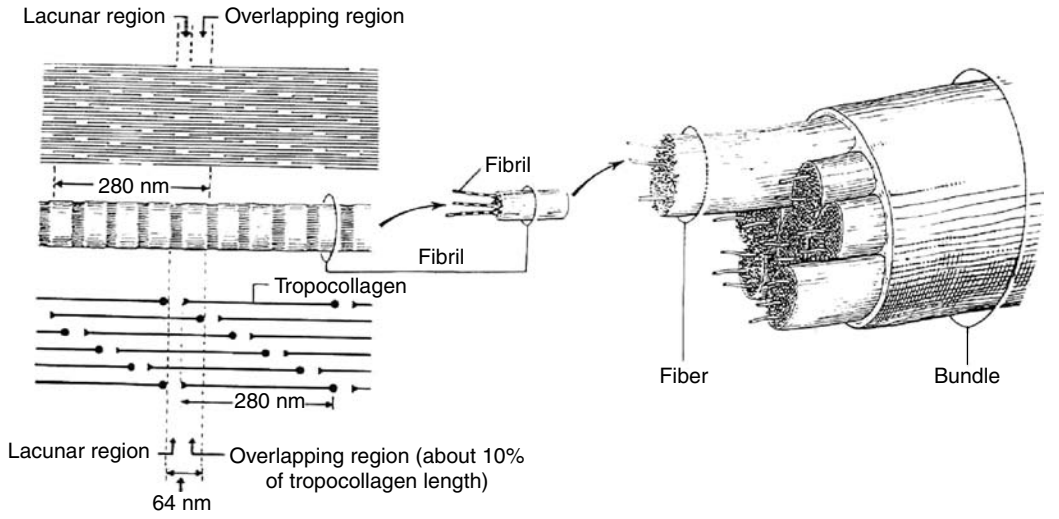


FIGURE 16.6 Formation of collagen fibrils. Tropocollagen units assemble in staggered, side-by-side arrays with head–tail overlap. Gaps and overlaps created in the staggered arrangement of tropocollagen units give rise to the appearance of light zones (lacunar regions) and dark zones (overlapping regions). Collagen fibrils encase muscle fibers, fiber bundles, and whole muscle. (Reprinted from Junqueira, L.C., J. Carneiro, and R.O. Kelley (1989). *Basic Histology*. Appleton & Lange, Norwalk, CT.)

levels of collagen and of lesser cross-linking relative to collagen in larger land animals. This accounts for collagen contributing less to tough texture in meat from small animals and fish.

The collagens are grouped according to the supramolecular structures that they form. These groups include (1) striated, fibrous; (2) nonfibrous, network forming; (3) microfibrillar or filamentous; and (4) fibril-associated. A few examples of types of collagen associated with muscle follow.

Types I, III, and V collagens are members of the striated collagen family. Examination of these collagens by electron microscopy reveals repeating bands every 64–70 nm, resulting from staggered side-by-side arrays of collagen molecules (Figure 16.6). These three collagen types are associated with epimysium that consists of only type I, and perimysium, which includes both types I and III with some type V [32]. Some of the older muscle literature refer to a heat-resistant, stromal protein fraction called reticulin that forms fibers associated with the perimysium. It is now clear that these fibers are type III collagen fibers, the abundance of which is correlated with meat toughness [33].The endomysium consists primarily of type IV collagen, which belongs to the network-forming collagen group [32]. Unlike the striated, fibrous collagens, type IV collagen forms a sheet-like appearance that resembles the structure of a chain-link fence. This structure arises from differences in amino acid sequence that prevent the side-by-side association observed in the striated collagen family. Small amounts of types I, III, and V are also associated with the endomysium.

The basic unit of collagen is called tropocollagen; it consists of three polypeptide chains that entwine around each other in a coiled-coil, superhelical fashion forming a linear molecule about 280 nm long and 1.4–1.5 nm wide (Figure 16.7). The chains may be identical or they may differ in amino acid sequence depending on the type of collagen. For example, type I collagen consists of two identical polypeptide chains termed α1(I) and one chain with a different amino acid sequence termed α2(I). Type III collagen consists of three identical chains: α1(III). By convention, the Arabic numbers are used to identify different collagen chains within a given type, whereas the Roman numerals refer to the collagen type. Accordingly, α1 chains from type I collagen differ from α1 chains from type III collagen.

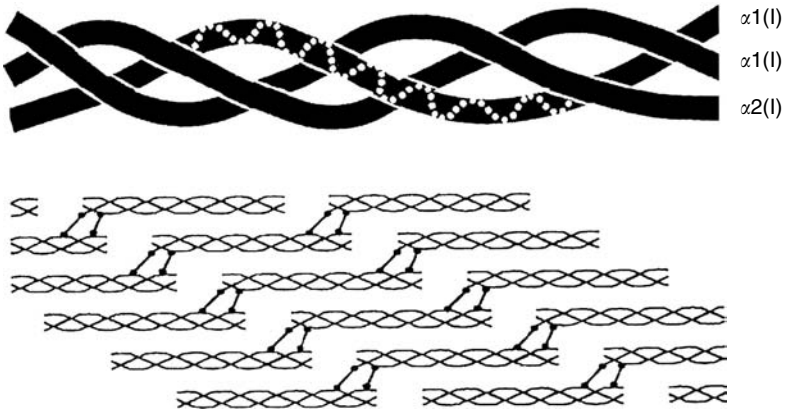


FIGURE 16.7 Schematic representation of the tropocollagen triple helix and cross-links between tropocollagen molecules in a collagen fibril. (Reprinted from Chiang, W., G.M. Strasburg, and T.M. Byrem (2007). In *Food Chemistry: Principles and Applications*, 2nd edn. Y.H. Hui (Ed.), Science Technology System, West Sacramento, CA.)

In type I collagen, an average polypeptide chain consists of approximately 1000 amino acid residues, with a characteristic repeating sequence throughout most of the chain of $(Gly–X–Y)_n$. Residue X in this sequence is often proline, and Y is often hydroxyproline or hydroxylysine. The latter amino acids are formed by posttranslational hydroxylation of proline and lysine by prolyl hydroxylase and lysyl hydroxylase, respectively. Overall, collagens generally contain approximately 33% glycine, 12% proline, 11% alanine, 10% hydroxyproline, 1% hydroxylysine, and small amounts of polar and charged amino acids. Tryptophan is notably absent from collagen; in fact, the absence of tryptophan in collagen preparations is sometimes used as a criterion for the purity of collagen preparations. The dominance of the amino acids identified above and the notable absence of most essential amino acids makes collagen a poor protein source in the human diet.

The characteristic amino acid sequence of collagen dictates the folding and assembly of the collagen family of proteins. The presence of glycine at the beginning of each triplet followed frequently by a proline residue gives rise to a highly extended polypeptide α-chain that forms an unique, shallow, left-handed helix. In type I collagen, three α-chains form a right-handed triple-helical coiled coil that constitutes the tropocollagen molecule (Figure 16.7). Structural studies indicate that the side chain of each glycine residue, that is, a hydrogen atom, is directed toward the center of the coiled-coil helix. Owing to the small size of the hydrogen atom compared with other amino acid side chains, glycine is the only amino acid whose side chain could be accommodated in such a structure. Moreover, each chain is slightly staggered with respect to the other two. This enables hydrogen bonding between the polypeptide amide hydrogen of a glycine residue with the carbonyl oxygen of the adjacent X residue on another chain. The presence of proline and hydroxyproline at frequent intervals along the sequence prevent the chain from adopting a classical α-helix because of constraints of allowable ϕ, ψ angles of these residues. Moreover, they lack amide hydrogen atoms that are characteristically involved in stabilization of α-helices. The hydroxyl groups of hydroxyproline and hydroxylysine are also thought to be stabilized by interchain hydrogen bonds. Thus, the secondary structure of collagen is an unusually extended, relatively rigid, distinctive helix among the proteins [34].

Collagen polypeptides are synthesized as precursors, termed pro-α chains. These precursor polypeptides include an N-terminal signal sequence that directs the polypeptide to the lumen of the endoplasmic reticulum of the fibroblast. Following the signal sequence at the N-terminal end as well as at the C-terminus is a series of additional residues collectively termed propeptides. Upon entry of the polypeptide into the lumen of the endoplasmic reticulum, selected proline and lysine residues are hydroxylated, and a few hydroxylysine residues are glycosylated. The pro-α chains then combine

to form the triple-stranded procollagen molecule. The propeptide sequences are believed to initiate formation of the procollagen molecule and they prevent the formation of large fibrils within the cell, which could not easily be secreted [25].

Procollagen is secreted to the extracellular matrix where proteinases cleave the propeptides from both ends of the molecule, forming tropocollagen which then begins self-assembly with other tropocollagen molecules to form fibrils. Tropocollagen molecules assemble in a staggered array through side-by-side associations stabilized primarily through hydrophobic and electrostatic interactions. The N-terminal 14 and C-terminal 10 residues of tropocollagen do not display the characteristic Gly–X–Y sequence found throughout most of the tropocollagen molecule; hence, these "telopeptide" regions do not form the characteristic collagen helix.

The telopeptide regions are involved in the formation of covalent intermolecular covalent cross-links between the individual chains (Figure 16.7). These cross-links provide critically needed stability and tensile strength to the supramolecular structure. There are four key residues involved in the initial cross-linking of tropocollagen chains: two lysine or hydroxylysine residues of the N-terminal telopeptides, and two lysine or hydroxylysine residues of the C-terminal telopeptides. The head-to-tail staggered arrangement of the tropocollagen molecules enables interactions of the N-terminal telopeptides with the adjacent C-terminal telopeptides.

The prerequisite step preceding cross-linking is oxidative deamination of the lysine or hydroxylysine residues to form allysine or hydroxyallysine, respectively, through the action of the enzyme lysyl oxidase. The subsequent cross-linking reactions occur spontaneously through aldol condensation or through the formation of Schiff base intermediates resulting from the condensation of an amino group from lysine or hydroxylysine with an aldehyde from allysine or hydroxyallysine. Several examples of these cross-linking reactions are shown in Figure 16.8. The divalent cross-links formed via these pathways are reducible by borohydride, and are thus termed "reducible cross-links."

As an animal matures, these divalent, reducible cross-links are converted to more stable, nonreducible, trivalent cross-links. Two types of mature cross-links have been characterized: hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) (Figure 16.9), the latter being present in only negligible amounts in muscle. HP is probably formed via condensation of two ketoamine cross-links (Figure 16.8), as evidenced by concurrent stoichiometric disappearance of reducible cross-links with appearance of the nonreducible forms [21]. In contrast to the reducible cross-links, nonreducible cross-links are very heat-stable, which has important implications for tenderness of meat. Moreover, additional cross-links that spontaneously form during maturation of the animal contributes to the increase in meat toughness, which is often found in older animals [21,35].

The cross-link content varies not only with age but also with muscle function (e.g., postural vs. locomotor), species, exercise, and treatment with growth promoters. Moreover, while both collagen content and degree of cross-linking are casually correlated with toughness of meat, it is evident that there is an additive effect of the two components. Less tender muscles, such as bovine biceps femoris, show high collagen concentrations as well as high concentrations of HP. In contrast, a relatively tender muscle such as longissimus dorsi possesses only half to two-thirds of the total amount of collagen and HP as the former muscle. Moreover, gluteus medius is high in collagen and low in HP content, whereas pectoralis major has low abundance of collagen but has high levels of HP. Both muscles are relatively tender. Thus, it appears that toughness is more strongly correlated with an additive effect of both collagen parameters [21].

16.3.4.2 Sarcoplasmic Proteins

Sarcoplasmic proteins are present in high abundance (25–30% of total muscle protein), and as the name implies, they are located in the sarcoplasmic (cytoplasmic) fraction of the muscle cell [17]. Many, but not all, of the proteins are enzymes involved in glycolysis, glycogen synthesis, and glycogenolysis (Table 16.5). One enzyme, glyceraldehyde phosphate dehydrogenase, constitutes as much as 20% of the sarcoplasmic fraction. Together, the next four or five most abundant glycolytic

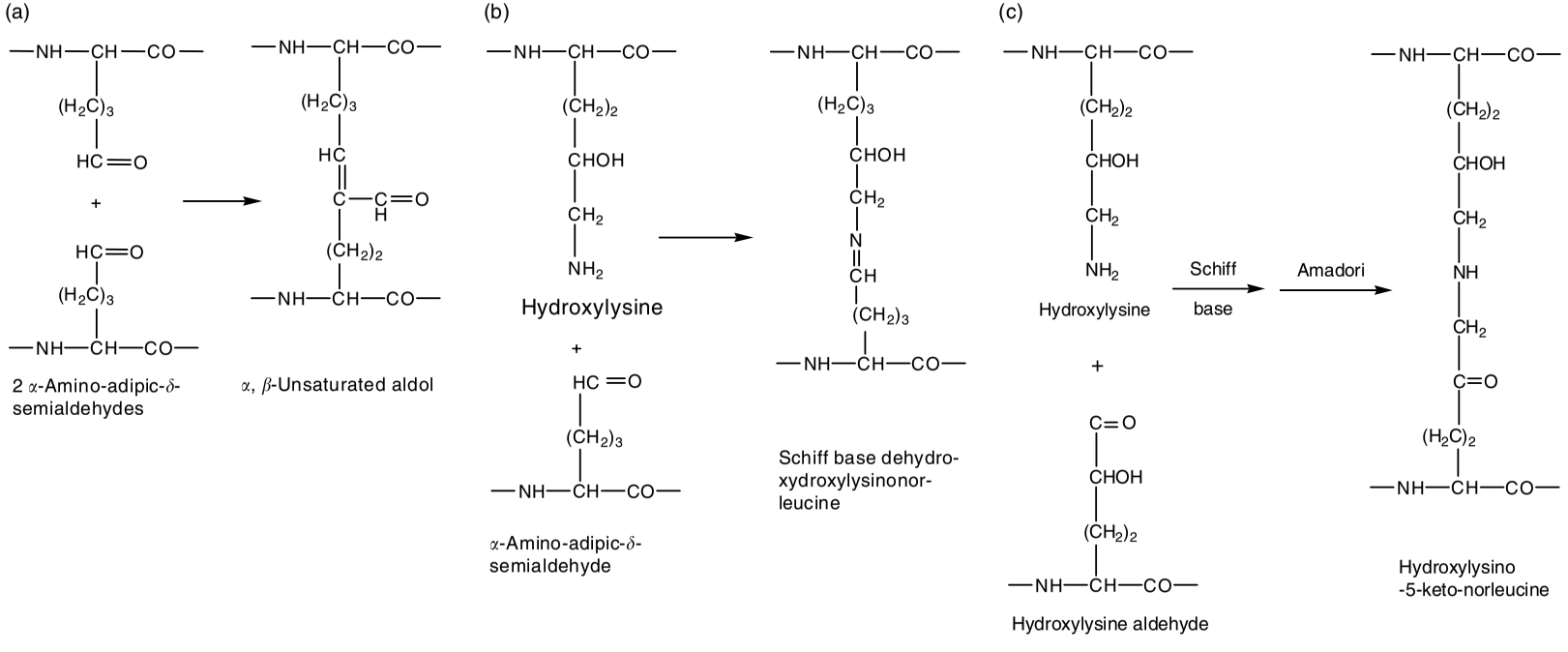


FIGURE 16.8 Mechanisms by which collagen side chains may form cross-links. (a) Aldol condensation of two allysine residues followed by loss of a water molecule; (b) Schiff base formation following condensation of hydroxylysine with allysine; and (c) Schiff base formation following condensation of hydroxylysine and hydroxyallysine. The Schiff base intermediate undergoes Amadori rearrangement to form hydroxylysino-5-keto-norleucine. (Reprinted from Foegeding, E.A., T.C. Lanier, and H.O. Hultin (1996). In *Food Chemistry*, 3rd edn. (O.R. Fennema, ed.), Marcel Dekker, Inc., NY.)

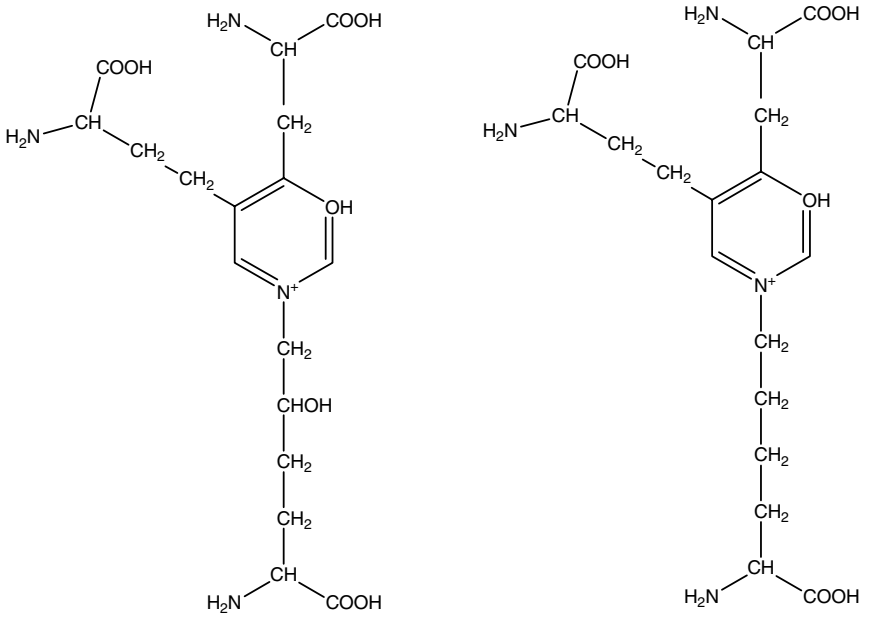


FIGURE 16.9 Structural formulas of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) collagen cross-links.

TABLE 16.5
Abundance of Sarcoplasmic Proteins in Muscle

Protein	mg/g
Phosphorylase B	2.5
Phosphoglucomutase	1.5
Phosphoglucose isomerase	1.0
Phosphofructokinase	1.0
Aldolase	6.0
Triose phosphate isomerase	2.0
$\alpha-$Glycerophosphate dehydrogenase	0.5
Glyceraldehyde phosphate dehydrogenase	12
Phosphoglycerate kinase	1.2
Phosphoglycerate mutase	1.0
Enolase	5
Pyruvate kinase	3
Lactate dehydrogenase	4
Creatine kinase	5
Adenylate kinase	0.5
AMP deaminase	0.2
Myoglobin	0.5–2.0

Source: Compiled from Scopes, R.K. (1970). Characterization and study of sarcoplasmic proteins (Ch. 22). In *The Physiology and Biochemistry of Muscle as a Food*, 2nd edn., (E.J. Briskey, R.G. Cassens, and B.B. Marsh, eds.), The University of Wisconsin Press, Madison, WI, pp. 471–492.

enzymes comprise over half of the total sarcoplasmic protein content. Other proteins include enzymes of the pentose shunt and auxiliary enzymes such as creatine kinase (the soluble fraction), AMP deaminase, the calpains, the oxygen storage protein, and myoglobin.

The abundance of some proteins found in the sarcoplasm may vary substantially depending on species, breed, muscle fiber type, age of the animal, and individual genetics. For example, myoglobin tends to be lower in abundance in young animals; thus, the pale color of veal compared to adult beef. Poultry breast muscles and many fish muscles have little redness because of the low levels of myoglobin, whereas poultry leg and thigh muscles are redder than breast muscles because of higher levels of myoglobin. At the other extreme, whale muscle contains the highest known levels of myoglobin; as much as 70% of the sarcoplasmic protein content of some whale muscles is myoglobin [17].

Another enzyme of great significance both in live muscle as well as in postmortem conversion of muscle to meat is creatine kinase. This enzyme is found both in the soluble sarcoplasmic protein fraction and as a component of the M-line protein matrix in the myofibril. Creatine kinase maintains stable levels of ATP for use by the muscle when subjected to intensive energy demands such as sprinting or lifting a heavy object [36]. ATP would quickly be depleted before glycolysis and oxidative metabolism could replenish the loss. Creatine phosphate (CrP) serves as a high energy reservoir compound that can donate its phosphate to ADP in the following reaction catalyzed by creatine kinase:

$$\mathrm{CrP} + \mathrm{MgADP} \rightarrow \mathrm{MgATP} + \mathrm{Cr}$$

In resting muscle, as glycolysis and oxidative metabolism restore ATP levels, the above process is reversed and some of the excess metabolic energy is converted from ATP to the reservoir form of CrP.

Two other proteins of note in the sarcoplasmic fraction are adenylate kinase and AMP deaminase. As ATP is utilized to meet energy demands, ADP is converted back to ATP by glycolysis, oxidative phosphorylation, and creatine kinase. Adenylate kinase is another enzyme that supports energy demands by synthesis of ATP through the following reaction:

$$2\mathrm{ADP} \rightarrow \mathrm{ATP} + \mathrm{AMP}$$

During periods of intense energy demands on muscle, as well as during the early phase of postmortem conversion of muscle to meat, ATP levels decline precipitously. This reaction becomes especially important when other sources of ATP generation become depleted. The other product, AMP, is deaminated by the action of AMP deaminase to inosine monophosphate (IMP) by the following reaction:

$$\mathrm{AMP} \rightarrow \mathrm{IMP} + \mathrm{NH_3}$$

IMP is further degraded to hypoxanthine that has a bitter off-flavor. Hypoxanthine has long been regarded as biochemical indicator of the postmortem age of fresh fish muscle.

Finally, several proteinases that are likely involved in muscle growth, maintenance, and postmortem muscle protein degradation exist in the muscle sarcoplasm. The role of these enzymes in aging and tenderization of meat will be described in Section 16.4.1. Another group of proteins is not soluble within the muscle sarcoplasm, but may be readily solubilized using various extraction methodologies. These proteins include some members of the intermediate and microfilaments comprising the cytoskeletal protein network in muscle.

16.3.4.3 Contractile Proteins

16.3.4.3.1 Myosin

The protein myosin serves as the molecular motor for muscle contraction. It is the dominant protein of the A-band, and at 45% of the myofibrillar protein content, it is the most abundant skeletal muscle

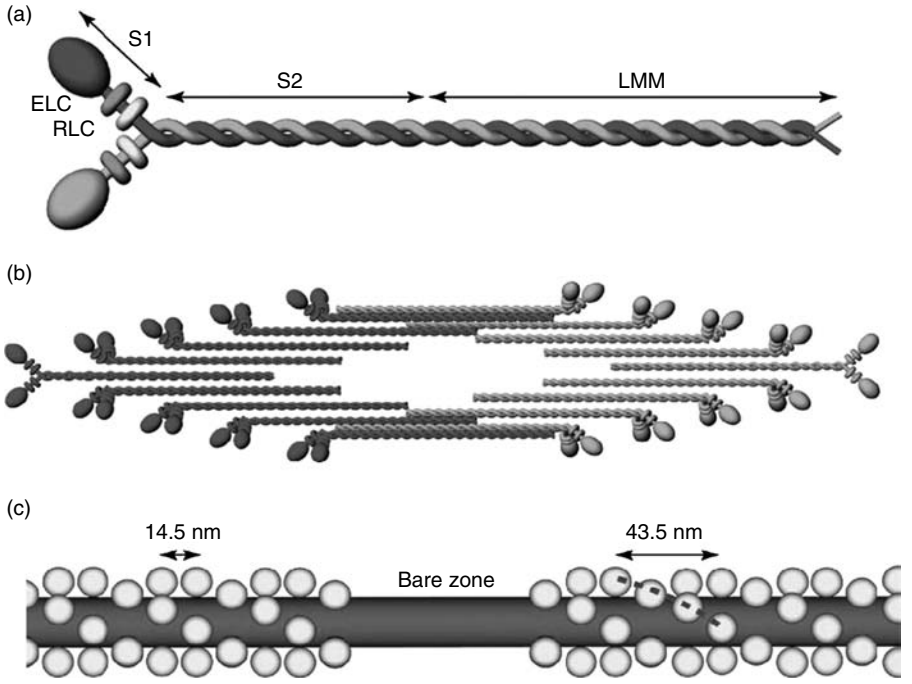


FIGURE 16.10 Schematic representation of the structural features of the myosin molecule and its assembly into thick filaments. (a) A functional myosin molecule consists of two heavy chains, each of which forms a globular head domain, and a rod-like α-helix. The helices of the two heavy chains form a rod-like coiled coil. Two light chains bind to the neck region of each heavy chain: the essential light chains (ELC) known as light chains 1 and 3, and the regulatory light chain (RLC), also known as light chain 2. Limited proteolysis of myosin yields heavy meromyosin (HMM) and light meromyosin (LMM). Limited proteolysis may also yield two subfragments of HMM: subfragment 1 (S1) consists of the head and neck domain of HMM, and subfragment 2 (S2) serves as a linker between the myosin head and the portion of the rod that is involved in filament formation. (b) Myosin molecules assemble in bipolar, staggered arrays to form thick filaments. (c) Myosin heads radiate from the thick filament shaft in a spiraling fashion. Parallel, adjacent myosin molecules are staggered by a distance of 14.5 nm; myosin heads are separated by a translational distance of 43.5 nm. (Reprinted from Craig, R. and J.L. Woodhead (2006). *Curr Opin Struct Biol* 16:204–212.)

protein [18]. A myosin molecule consists of six subunits: two "heavy chains" of $M_r \sim 220,000$ and four "light chains" that vary in mass from about 16,000 to 20,000 [37]. These six subunits combine to form a quaternary structure with a molecular mass of ~500,000 with the general appearance of two globular heads projecting from the end of a stick (Figure 16.10a). The N-terminal end of each heavy chain folds into the structure referred to as the myosin head, while the C-terminal portion (60% of the residues) consists of a sequence motif that gives rise to a long α-helix. The helices of two heavy chains intertwine to form a rod-like coiled coil. Two myosin light chains bind to each myosin head. The light chains of myosin consist of two alkali-extractable polypeptides, referred to as light chain 1, LC1 ($M_r \sim 20,900$), and light chain 3, LC3 ($M_r \sim 16,600$), respectively. Myosin light chain 2 is sometimes referred to as the (5,5′-dithiobis)-2-nitrobenzoic acid (DTNB)-light chain because treatment of myosin with DTNB removes this protein ($M_r \sim 18,000$) from the myosin head. Each myosin head contains one of the alkali-extractable light chains and one DTNB light chain. The DTNB light chain is sometimes called the regulatory light chain, because it is required for myosin-based regulation of muscle contraction in molluscan muscles and smooth muscles of higher organisms. Likewise, LC1 and LC3 are sometimes referred to as essential light chains.

The head and rod portions have distinctly different, important functions. Under physiological conditions, myosin molecules coalesce through staggered, side-by-side associations of the rod portions of each molecule (Figure 16.10b). Thus, the myosin rod domain provides the structural basis for the formation of thick filament. The thick filaments are arranged in bipolar fashion such that the origin of the filament is the M-line, and myosin molecules proceed in opposite directions away from the M-line. The myosin heads project radially from each thick filament shaft, and are directed toward the thin filaments (Figure 16.10c). The top of the myosin head binds to actin, the backbone protein of the thin filament. The myosin head also contains an ATP-binding site which serves as the molecular motor that drives muscle contraction.

Study of the functional nature of the different myosin protein domains was facilitated by the discovery that brief proteolytic treatment of myosin with enzymes such as trypsin or papain resulted in fragments of the protein that were more suitable for study under physiological conditions [38]. Two primary products of this limited proteolysis were obtained. One fragment, called heavy meromyosin (HMM), consisted of two myosin heads plus a short portion of the myosin rod emanating from the head. HMM maintained the actin-binding and ATP-hydrolytic activities of the parent myosin molecule. The remaining rod portion was called light meromyosin (LMM); it retained the molecular assembly determinants that enable the assembly of individual myosin molecules into thick filaments. Subsequently it was shown that further proteolytic treatment of HMM resulted in cleavage at the head–tail junction, thus yielding two products. The product consisting of the myosin head was called subfragment 1 (S1), and the short tail portion was called subfragment 2 (S2) (Figure 16.10a). Subfragment 2 possessed neither the self-assembly property nor the molecular motor activity of the parent molecule. Thus, it appeared to serve as a linker segment that separates the myosin head from the thick filament shaft [39].

Myosin subfragment 1 has been crystallized and its molecular structure has been determined at high resolution [40]. Among the many features observable in the structure of S1 are two prominent clefts. One pocket serves as the ATP-binding site, and the other cleft is the actin-binding site (Figure 16.11). These two functions constitute the essential features of the molecular motor activity of the myosin molecule. In addition, the structure shows a long α-helical segment that connects the myosin head (S1) to the myosin rod (S2). This helix serves as a lever arm at which the conformational change associated with myosin head movement takes place during muscle contraction.

16.3.4.3.2 Actin

At approximately 20% of the myofibrillar protein content, actin is the second most abundant protein in muscle. The actin monomer, called globular actin or G-actin, is a single polypeptide chain with a molecular mass of 42,000 and a single adenine- nucleotide-binding site. The term G-actin is rooted in the historical view that the monomer was approximately spherical. When the high-resolution crystal structure of G-actin was determined, it became clear that molecule is actually shaped rather like a peanut shell with two large domains subdivided into two additional sub-domains [41]. Nevertheless, the term G-actin persists to describe the actin monomer.

G-actin remains monomeric at very low ionic strength. However, under conditions approaching physiological ionic strength coupled with the presence of MgATP, actin polymerizes in "head-to-tail" fashion to form double-stranded, coiled-coil, thin filaments called filamentous actin or F-actin [42]. One end of each filament is anchored in the Z-disc, and the filaments project toward the M-line located at the center of the sarcomere (Figure 16.3a). Because of the directional nature of the thin filaments, actin filaments on opposite sides of the M-line are directed toward each other like arrows within the sarcomere. The lengths of the thin filaments are remarkably precise at approximately 1 μM. It is believed that other thin filament proteins including nebulin, tropomodulin, and CapZ protein play key roles in controlling the size of individual thin filaments [37].

Actin plays a dual role in the myofibril. It binds myosin during muscle contraction, forming actomyosin crossbridges between the two filaments. Binding of actin to myosin activates the ATPase activity of myosin, causing myosin to act as a molecular motor that pulls thin filaments past the thick

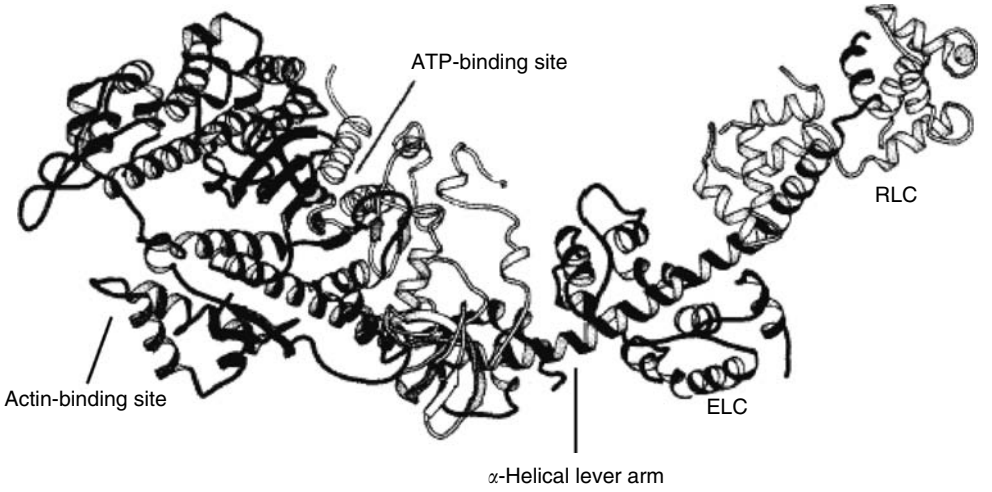


FIGURE 16.11 Representation of the three-dimensional structure of myosin subfragment 1. Two clefts are prominent in the myosin head: an actin-binding site that serves as the domain for crossbridge formation, and a nucleotide-binding site where ATP undergoes hydrolysis to yield mechanical energy to support muscle contraction. The structure also indicates a long α-helix that serves as a binding domain for the light chains, and as a lever arm on which myosin conformational changes occur during contraction and relaxation. Abbreviations: RLC, Regulatory light chain; ELC, Essential light chain. (Reprinted from Rayment I., C. Smith, and R.G. Yount (1996). *Annu Rev Physiol* 58:671–702.)

filaments to shorten the sarcomere. Second, actin forms the backbone for binding tropomyosin and troponin, two proteins that act in concert to regulate actin–myosin interaction in response to changes in calcium levels.

16.3.4.4 Regulatory Proteins

16.3.4.4.1 Tropomyosin

Tropomyosin together with troponin constitute the regulatory switch that turns muscle contraction on or off within the sarcomere [43]. Tropomyosin consists of two α-helical subunits ($M_r \sim 37,000$ Da) that intertwine to form a long, rod-like, coiled-coil protein with a molecular mass of ~74,000 Da. Two different tropomyosin isoform subunits, termed α- and β-tropomyosin, are found in skeletal muscle. These subunits may combine in various ways to form homodimers ($\alpha\alpha$ or $\beta\beta$) or heterodimers ($\alpha\beta$) [44]. The actual amounts of each isoform expressed in myofibers depend on muscle fiber type (fast vs. slow), muscle type (i.e., skeletal, cardiac, or smooth), stage of development, and species. Moreover, various alternative splicing patterns of tropomyosin generate several additional isoforms that presumably provide an additional level of modulation of contractile properties of different types of myofibers [44].

Like actin, tropomyosin polymerizes at physiological ionic strength in head-to-tail fashion, with a small overlap of about 8–11 amino acids at each end of a tropomyosin molecule. An individual tropomyosin molecule is approximately 42 nm long and spans seven actin monomeric units (Figure 16.3c). The filamentous strand of tropomyosin binds to the actin backbone at specific sites along each actin monomer, near the grooves of the thin filament double helix. In this position, tropomyosin blocks the myosin-binding sites on the outer domain of the actin filament when muscle is at rest [44].

16.3.4.4.2 Troponin

The troponin complex, which constitutes 5% of the myofibrillar protein, is a heterotrimer that binds to tropomyosin (Figure 16.12). Working in concert with tropomyosin, troponin responds to changes

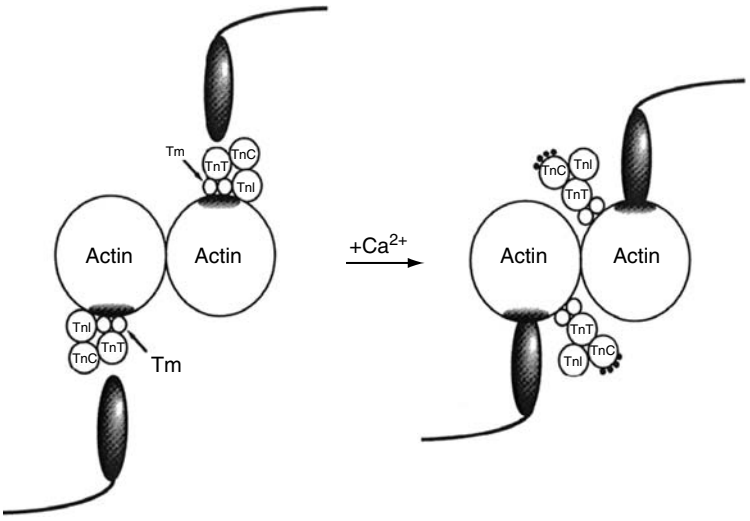


FIGURE 16.12 Regulation of contraction and relaxation by the tropomyosin–troponin complex. When muscle is in the resting state, tropomyosin sterically blocks myosin heads from binding to the myosin-binding site (shaded region) on the actin filament. When calcium binds to troponin-C, the troponin complex undergoes a conformational change that causes tropomyosin to shift deeper into the actin groove, exposing myosin-binding sites and enabling myosin crossbridge formation with actin. (Reprinted from Chiang, W., G.M. Strasburg, and T.M. Byrem (2007). In *Food Chemistry: Principles and Applications*, 2nd edn. Y.H. Hui (Ed.), Science Technology System, West Sacramento, CA.)

in calcium ion concentration to control actin–myosin interaction, and thus, contraction or relaxation of muscle [43].

Troponin-C (TnC) has an M_r of ~18,000 and is the calcium-binding subunit of troponin [45]. TnC consists of four Ca^{2+}-binding sites; two are high affinity Ca^{2+}-binding sites ($K_d \sim 10^{-9}$ M) that also bind Mg^{2+}. These two binding sites are located in the C-terminal half of the molecule. Two lower affinity calcium-binding sites ($K_d \sim 10^{-6}$ M) are in the N-terminal half of the molecule. These two sites are specific for binding Ca^{2+}. In resting muscle, the two low-affinity binding sites are believed to be empty because of the low [Ca^{2+}] ($<10^{-7}$ M), whereas the high-affinity sites are likely to be occupied by Mg^{2+} because of its high-intracellular concentration relative to Ca^{2+} [46]. When a motor neuron initiates muscle contraction, [Ca^{2+}] concentrations rise to $>10^{-5}$ M; some of the calcium ions bind to low-affinity sites of TnC, triggering a conformational change in the protein that is transmitted through the other troponin subunits to tropomyosin.

Troponin-T binds the troponin complex to tropomyosin, and troponin-I inhibits actomyosin ATPase activity. The troponin complex binds near the head–tail junction of a tropomyosin filament at a ratio of one troponin per tropomyosin molecule [43].

16.3.4.5 Structural Proteins

16.3.4.5.1 Titin

Titin (also known as connectin) is the third most abundant protein of muscle myofibrils. With over 38,000 amino acid residues and an M_r of ~4,200,000, titin is the largest single polypeptide chain known. Yet despite titin's abundance in muscle, it was discovered relatively recently by K. Wang and coworkers in 1979 [47]. Ironically, this protein escaped notice because it is so large: titin's extremely high molecular mass limited its mobility on conventional polyacrylamide gel systems used to characterize muscle proteins. Use of low-acrylamide concentrations in sodium dodecyl sulphate–polyacrylamide gels created much larger pore sizes in gels that enabled titin to

migrate into the gels, thus leading to the discovery of this protein as well as another large protein, nebulin.

Titin is a flexible, elastic filamentous protein of about 1 μM in length that spans one-half of a sarcomeric unit (Figure 16.3a). The N-terminus of titin is located in the Z-disc, and the C-terminus is anchored in the M-line. The titin domain in the I-band region functions as a molecular spring that maintains the precise structural arrangement of thick and thin filaments, and gives rise to passive muscle stiffness. The extensible region of titin in the skeletal muscle is composed of tandemly arranged immunoglobulin-like domains, and a PEVK-segment that is abundant in proline (P), glutamate (E), valine (V), and lysine (K) [48]. In addition to these segments, the extensible region of cardiac titin also contains a unique 572-residue sequence that is part of the cardiac-specific N2B element [49]. These segments have distinct bending rigidities and as a result, stretching of slack sarcomeres initially gives rise to extension of tandem Ig segments followed by extension of PEVK segment. When muscle relaxes, titin restores muscle to its resting sarcomere length. Titin may also serve as a template upon which myosin molecules form thick filaments because titin is one of the earliest genes expressed in muscle protein synthesis as muscle is formed during embryogenesis [50].

16.3.4.5.2 Nebulin

Like titin, nebulin was discovered rather recently because of its unusually large size ($M_r \sim 800,000$). It is present in amounts comparable to that of tropomyosin and troponin. Nebulin is associated with the thin filaments of skeletal muscle, with the C-terminal end partially inserted into the Z-discs, and the N-terminal end extending to the pointed ends of the thin filaments. There is also an amino acid sequence super-repeat in the nebulin sequence, analogous to that of titin. However, nebulin is inextensible (unlike titin), and its unique structural properties have made it a prime candidate to act as a molecular ruler for specifying the precise lengths of the thin filaments [51]. It may also be involved in signal transduction, contractile regulation, and myofibril force generation [52].

16.3.4.5.3 α-Actinin

A major component of the Z-disc is α-actinin ($M_r \sim 97,000$) which functions as an actin-binding protein. α-Actinin contains three major domains: a globular N-terminal actin-binding domain, a central rod domain, and a C-terminal domain that bears similarity to the calcium-binding protein calmodulin [53]. The rod domains of α-actinin monomers interact to establish antiparallel dimers that are capable of cross-linking actin and titin filaments from neighboring sarcomeres. In fact, α-actinin has the capacity to associate with numerous protein partners at the Z-disc. These protein interactions provide tensile integrity to the Z-disc, and may also serve as an additional docking site for other Z-disc-associated proteins (Figure 16.5).

16.3.4.5.4 CapZ and Tropomodulin

Actin filament formation is a dynamic process, and regulation of this process during myofilaments assembly is a key factor in maintaining their uniform filament lengths. Proteins that bind to and cap the thin filaments block filament elongation and shortening. CapZ, also known as β-actinin, is a capping protein involved in nucleation and stabilization of actin filaments. It is a heterodimer containing α ($M_r \sim 36,000$) and β ($M_r \sim 32,000$) subunits, both of which are required for capping [37,54]. In striated muscle, CapZ is localized to Z-discs where it binds α-actinin, and likely forms an anchoring complex for the thin filaments. Tropomodulin ($M_r \sim 40,000$) caps the pointed ends of the thin filaments which, in turn, modulate thin filament lengths [37].

16.3.4.5.5 Desmin

Desmin ($M_r \sim 55,000$) is the dominant protein of the intermediate filaments (10 nm diameter) that maintains the structural integrity of most cells. In muscle, desmin filaments are found at the periphery of Z-discs, and it appears that these filaments serve to cross-link adjacent myofibrils and to link the myofibrillar Z-discs to the SL [55] (Figure 16.5).

16.3.4.5.6 Filamin

Three filamins (α, β, and γ) ($M_r \sim 300,000$) have been identified in muscle. They share a similar molecular structure: an N-terminal actin-binding head followed by 24 immunoglobulin-like domains. γ-Filamin (also known as ABP-280) is the muscle-specific filamin isoform. Filamin is localized at the periphery of the Z-disc and provides a critical link between the SL and sarcomeric cytoskeleton. Since γ-filamin exists at the very early stages of developing Z-discs, it is suggested that this filamin isoform might be involved in the formation of sarcomeric Z-discs [56].

16.3.4.5.7 C-protein and H-protein

C-protein ($M_r \sim 140,000$) and H-protein ($M_r \sim 58,000$) are myosin-binding proteins. They are both distributed in the C-zone (middle third of each half of the A-band) of the thick filament, forming a series of transverse stripes spaced 43 nm apart, although H-protein is also found outside this zone [57]. C-protein is found in approximately a 1:8 molar ratio with myosin heavy chains and interacts with both the myosin and titin filaments. C-protein and H-protein may function to link and/or align the thick filaments in the A-band [58].

16.3.4.5.8 Myomesin and M-line proteins

The main protein of the M-line is myomesin, a polypeptide of about 185,000 Da [37]. In addition to serving as scaffolding for binding of titin and myosin, it may be involved in maintaining structural integrity of thick filaments. Another component of the M-line is creatine kinase, an enzyme involved in regeneration of ATP in muscle as described in Section 16.3.4.2.

16.3.4.6 Proteins of the Sarcoplasmic Reticulum and Sarcolemma

The membranes of the various organelles of muscle—nuclei, mitochondria, SL, and so forth—contain proteins that serve functions relevant to the specific needs of the organelles and of the cells. The proteins of the SR merit special attention because of their role in storage, transport, and release of calcium ions in the myofiber. Because calcium ions trigger muscle contraction and regulate key glycolytic steps, these proteins play key roles in muscle activity of the live organism. The proteins of the SR may also serve as key determinants of meat quality, depending on the extent to which they control sarcoplasmic calcium concentrations in the initial time period postmortem. Although there are many proteins that are found in the SR and SL, our focus here is on three proteins. Two of these proteins, the ryanodine receptor (RyR) and the dihydropyridine receptor (DHPR), comprise the essential elements of the calcium-release mechanism responsible for initiating muscle contraction. The third protein, the calcium pump, is involved in relaxation of muscle.

The DHPR is an intrinsic membrane protein complex embedded in the T-tubules that serves as a sensor for the change in voltage across the membrane when muscle contraction is initiated [59]. The DHPR consists of four different subunits, referred to as α_1, β, γ, and $\alpha_2\delta$ subunits. The α_1 subunit forms a calcium channel, which is responsible for the voltage-sensor function, and contains the binding site for a class of pharmaceutical compounds called dihydropyridines that modulate the protein's function. The roles of the other protein subunits are less clear, but there is evidence supporting their role in modulating the process of excitation–contraction coupling [60]. The function of DHPRs in various tissues depends in large part on the isoform expressed in the tissues. For example, the cardiac-muscle-specific α_1 isoform allows inward diffusion of calcium ions from the extracellular milieu, whereas the skeletal muscle isoform is largely inactive as a channel.

A portion of the SR called the terminal cisternae is closely apposed to the T-tubule. This portion of the SR is enriched in a protein called the SR calcium-release channel or RyR, so named because it binds a toxic plant alkaloid (ryanodine) very specifically and with high affinity. The RyR family consists of a series of homologous proteins sometimes referred to as RyR1, RyR2, and RyR3. In mammals, RyR1 is the skeletal muscle isoform, while RyR2 is the dominant form of cardiac muscle.

RyR2 is also found in other tissues including brain. RyR3 is found in various mammalian nonmuscle tissues and in trace amounts in skeletal muscle. In avian, piscine, and amphibian species, RyR3 is found in most skeletal muscles in approximately equal abundance with that of RyR1 [61]. RyRs function as channels or transmembrane conduits for diffusion of calcium ions from within the lumen of the SR, where the concentration is several millimolar, to the sarcoplasm where the resting muscle calcium concentration is $<10^{-7}$ M.

The RyR is an extremely large protein that consists of four identical subunits, each with a molecular weight of approximately 550,000, resulting in a functional ion channel with a molecular mass of ~2,200 kDa. Several other proteins are tightly associated with the RyR and modulate its function [11]. More than 80% of the RyR mass is on the cytoplasmic side of the SR membrane, forming a structure sometimes referred to as junctional foot that spans the gap between the terminal cisternae of the SR and the T-tubule. There are numerous lines of evidence suggesting skeletal muscle DHPRs and RyRs are in physical association [12]. Electron micrographs show that DHPRs assemble into groups of four units called a "tetrad." Overlays of DHPR tetrads in the T-tubule with SR junctional foot proteins (RyRs) suggest that every other RyR interacts with a tetrad; conversely, every other RyR not coupled with a DHPR (Figure 16.13) [62]. Recent evidence suggests that in birds, fish, and amphibians, RyR3, which is generally found in equal abundance with RyR1, is confined to the periphery of the SR/T-tubule junction and, thus, does not interact with the T-tubule or with the DHPR tetrad [63].

The DHPR and RyR work in concert with their associated regulatory proteins to affect Ca^{2+}-release into the sarcoplasm that triggers muscle contraction. When the neuromuscular contraction signal ceases, these proteins return to their "resting" state and the sarcoplasmic calcium concentration is restored by the action of the calcium pump protein. This protein is located primarily in the longitudinal SR, that is, the portion of the SR distal to the terminal cisternae. Using ATP as an energy

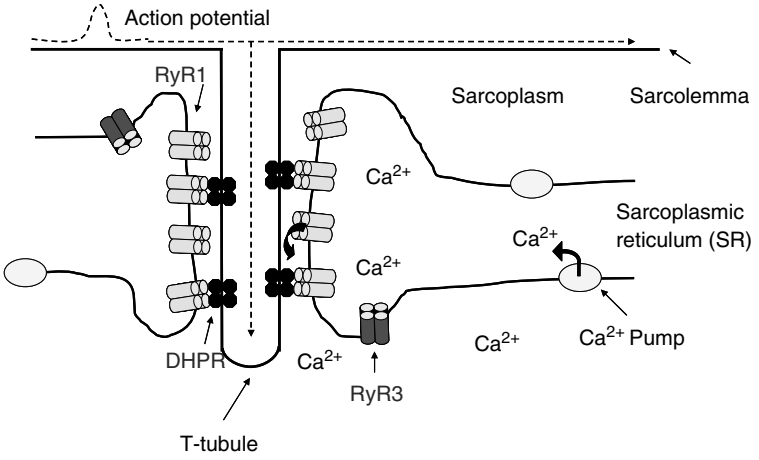


FIGURE 16.13 Schematic representation of the calcium release and uptake mechanisms in skeletal muscle. Dihydropyridine receptors (DHPRs) embedded in the T-tubule membrane serve as sensors of the action potential initiated by a motor neuron. Ryanodine receptors (RyR1 and RyR3) located in the SR serve as calcium channel proteins that open to allow calcium ions to diffuse from the SR to the sarcoplasm during contraction. Calcium pump proteins lower the sarcoplasmic calcium ion concentration by translocating calcium into the SR after RyR pores close. In mammalian skeletal muscle, every other RyR1 is coupled to a DHPR tetrad. RyR3 is localized in the peripheral region of the T-tubule, and does not associate with the DHPR. RyR3 is present in very low abundance in mammalian skeletal muscle, but is approximately equal to that of RyR1 in most avian, piscine, and amphibian muscles.

source, the calcium pump protein transports two Ca^{2+} ions against the concentration gradient across the SR membrane and into the lumen of the SR for every ATP molecule hydrolyzed [64].

16.3.5 Excitation–Contraction Coupling

A detailed understanding of the mechanism of muscle contraction may, at first glance, seem irrelevant to meat quality. The difference between live muscle tissue and meat is quite literally the difference between life and death. However, upon slaughter of an animal, muscle tissue goes through a series of transitional reactions (described in Section 16.4) known as conversion of muscle to meat. The biochemical events associated with this transition involve exactly the same mechanisms of muscle contraction and the supporting physiological and biochemical reactions of live muscle, all of which are significant contributing determinants of meat quality.

When the muscle cell is at rest, there is a voltage difference across the SL of about −90 mV and the intracellular Ca^{2+} concentration is very low ($<10^{-7}$ M). In the myofibrils, the tropomyosin is situated on the actin filament in a position so as to sterically prevent myosin–actin crossbridge formation. Upon stimulation of a myofiber by a motor neuron, the muscle cell is depolarized at the neuromuscular junction. This voltage change travels along the SL, entering the interior of the muscle via the T-tubule. The DHPR responds to the electrochemical transient across the membrane by undergoing a conformational change that is transmitted across the T-tubule/SR junction to the RyR located in the SR terminal cisternae. The RyR, in turn, responds by opening its channel pore. Calcium ions flow from the lumen of the SR, where the concentration is $>10^{-3}$ M, to the sarcoplasm, thus raising the sarcoplasmic concentration of calcium ions over 100-fold to $>10^{-5}$ M. Ca^{2+} binds to TnC, resulting in a conformational change in the troponin complex that is transmitted to tropomyosin. This results in a shift of the position of tropomyosin along the actin filament. Tropomyosin shifts deeper into the thin filament groove, thereby exposing myosin-binding sites on the actin monomers and enabling actin–myosin crossbridge formation (Figure 16.12) [24].

At this point in the muscle contraction cycle (illustrated in Figure 16.14), the nucleotide-binding pocket of the myosin head contains ADP and inorganic phosphate (P_i), which are products of

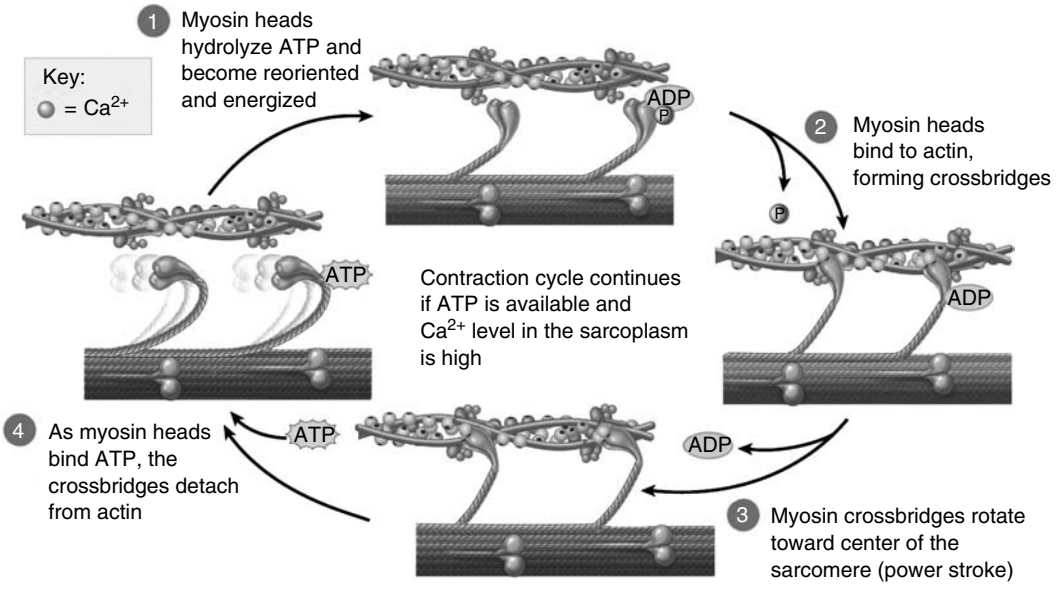


FIGURE 16.14 Crossbridge cycling during muscle contraction. (Reprinted from Tortora, G.J. and B. Derrickson (2006). *Principles of Anatomy and Physiology*. John Wiley and Sons, Inc., Hoboken, NJ.)

hydrolysis of ATP. Exposure of the myosin-binding sites on the actin filament enables weak binding of the myosin head to the thin filament, with the myosin heads binding in an approximately perpendicular orientation relative to the thick filament axis. Upon binding to actin, the myosin head releases P_i, causing a small conformational change in myosin, and strengthening the binding of myosin to actin. This is immediately followed by a large conformational change in the myosin head called the power stroke in which the myosin heads pull the actin filament along the thick filament in a rowing-like motion toward the M-line. ADP is then released from the myosin head, and ATP then binds to the empty nucleotide-binding site that triggers release of the myosin head from actin. Hydrolysis of ATP within the myosin head results in a conformational change that "cocks" the myosin head so that it is again approximately perpendicular to the thick filament, and the reaction series undergoes another cycle of contraction [65]. Because the actin and myosin filaments within a sarcomere are oriented toward the M-line, each contraction cycle results in shortening the distance between the Z-discs, thereby affecting the contraction of muscle (Figure 16.4 and 16.14) [66].

The myosin crossbridge cycling of attachment, power stroke, and detachment continues to maintain tension or contraction as long as neuronal stimulation of the myofiber continues. Upon cessation of the stimulus, sarcoplasmic Ca^{2+} is reduced to resting muscle concentrations by the action of the calcium pump protein. As the sarcoplasmic calcium ion concentration is lowered, TnC is depleted of Ca^{2+} from the low-affinity Ca^{2+}-binding sites, resulting in a conformational change in TnC reverting to the structure associated with the relaxed state of muscle. This conformational change causes tropomyosin to move back to its resting-state position on the actin filament, thereby blocking myosin–actin crossbridge formation, resulting in muscle relaxation.

It is important to recognize the multiple roles of ATP in muscle contraction and to recognize its critical role in conversion of muscle to meat. As the dominant energy source for the cell, hydrolysis of the ATP phosphodiester bond is converted to mechanical energy by myosin resulting in muscle contraction. In addition to the other myriad chemical reactions requiring ATP, the role of ATP in support of the function of the calcium pump is pivotal to the conversion of muscle to meat. As ATP levels decline, the ability of the calcium pump to sequester sarcoplasmic Ca^{2+} is compromised. Finally, it should be evident that after death of the animal, ATP levels will eventually drop to the point at which there is no longer sufficient ATP to bind to myosin heads, which would keep myosin dissociated from actin. The position at the end of the power stroke in which the myosin head is bound to actin is sometimes referred to as the "rigor complex."

16.3.6 Muscle Fiber Types

Skeletal muscle comprises several different types of muscle fibers that display differences in function, including speed of contraction (fast vs. slow) and supporting metabolism (oxidative vs. anaerobic). The muscle fiber types have great adaptive potential and their phenotypic profiles are affected by the type of neuron innervating the muscle fiber, neuromuscular activity, exercise/training, mechanical loading/unloading, hormones, and aging [67]. These fiber types can be defined using histochemical, biochemical, morphological, or physiological characteristics; however, classification of muscle fibers by different techniques does not always agree. Initially, muscles were classified as being fast-twitch (type I) or slow-twitch (type II), on the basis of the speed of shortening of individual fibers [68]. This classification also corresponded to a morphological difference, with the fast-twitch muscles appearing white in some species, and the slow-twitch muscles appearing red. The redness of the fiber correlates with high amounts of myoglobin, which provides a ready source of oxygen in support of oxidative metabolism. The slow-twitch, red fibers generally contain greater amounts of mitochondria and lipids as fuel for oxidative metabolism, whereas fast-twitch fibers are more equipped for anaerobic metabolism fueled by carbohydrates. Later, the type I and type II muscle fibers were reclassified with a bifunctional nomenclature based on the contractile properties and oxidative capacity of the fibers. More specifically, "fast-twitch oxidative" (FOG) muscle contracts with

a faster contractile speed compared with "slow-twitch oxidative" (SO), but has a higher oxidative capacity than "fast-twitch glycolytic" (FG) muscle.

With the advent of immunohistochemical staining methods capable of differentiating myosin heavy chain isoforms, fiber types could be further categorized into type I "slow- red" and type IIa "fast-red," while type IIb "fast-white" was categorized into types IIx and IIb in rodents, and IIx, but not IIb, is expressed in humans [69]. A good correlation exists between type I and SO fibers. However, the correlations between type IIa and FOG and type IIb and FG fibers are more varied. The type IIb fibers do not always rely primarily on anaerobic/glycolytic metabolism, nor do the type IIa fibers always rely primarily on aerobic/oxidative metabolism [70]. With the development of new molecular methods, it is evident that more specific classifications of muscle fiber types will be developed.

16.4 CONVERSION OF MUSCLE TO MEAT

Live muscle tissue has an extensively developed metabolic system designed to support the specific function of muscle, which is to convert chemical energy into mechanical energy. The immediate source of chemical energy is ATP, and the supporting metabolic reactions are geared in large part toward sustaining levels of ATP needed for contraction as well as for maintaining cellular homeostasis. Circulation of blood to the muscle tissue to deliver oxygen and energy substrates and to remove carbon dioxide and metabolic end products is clearly critical to supporting this metabolic machinery.

Upon slaughter of an animal, the initial step in the conversion of muscle to meat is the cessation of blood flow to muscle that occurs at slaughter. Although physiological death of the animal generally occurs within moments after slaughter, the various organs of the body including muscle draw on reserve mechanisms in a futile effort to maintain cellular homeostasis. Without continuous oxygen delivery to the muscle, the myofiber utilizes remaining oxygen bound to myoglobin to support aerobic metabolism. Quickly thereafter, anaerobic glycolysis becomes the dominant metabolic pathway for the generation of ATP. As metabolic end products accumulate and substrates become exhausted, the synthesis of ATP can no longer match the rate at which it is being hydrolyzed. Sarcoplasmic Ca^{2+} concentrations can no longer be sustained at resting state levels, while other ion pumps requiring ATP, such as the Na^{+}–K^{+}–ATPase, become inoperative. The decline of ATP also results in the stiffening of muscle known as rigor mortis; this results from the lack of sufficient ATP to dissociate myosin from actin during contraction. Eventually, endogenous enzymatic activity results in partial degradation of the structure of the myofilament framework. The time course of this series of reactions varies widely from species to species, and as will be seen later, even within a species.

There are few primary reserves of energy that fuel the immediate needs of muscle in the early postmortem state. CrP serves as a rapidly accessible reservoir of high-energy phosphate; as ATP is hydrolyzed to meet the demands of muscle contraction, creatine kinase transfers the phosphate moiety from CrP to ADP, thereby regenerating ATP. The enzyme adenylate kinase (described in Section 16.3.4.2) probably also contributes to the generation of ATP at this point. However, in postmortem muscle, these sources of ATP synthesis are soon exhausted. Glycogen is generally a greater resource for synthesis of ATP. The enzyme phosphorylase cleaves monomeric glucose units from glycogen, yielding glucose-1-phosphate that subsequently enters the glycolytic pathway to generate ATP. In the absence of oxygen, pyruvate is further converted to lactic acid that accumulates in the muscle.

As can be seen in Figure 16.15, the initial generation of ATP from CrP and from glycogen is nearly equal to the rate of ATP utilization, thus sustaining the ATP concentration in the range of 5 mM. As long as ATP levels remain high, the physiological requirements of the muscle are generally supported and the physical characteristics of the tissue during this early stage of conversion of muscle to meat (delay phase) remain similar to those of live muscle. Most notably, the muscle remains

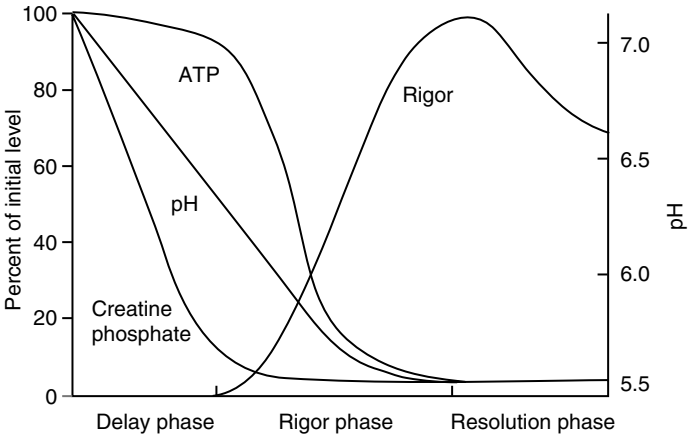


FIGURE 16.15 Schematic representation of postmortem changes in ATP concentration, creatine phosphate concentration, pH, and rigor development as a function of time. (Reprinted from Chiang, W., G.M. Strasburg, and T.M. Byrem (2007). In *Food Chemistry: Principles and Applications*, 2nd edn. Y.H. Hui (Ed.), Science Technology System, West Sacramento, CA.)

pliable and undergoes lengthening when subjected to stretch. However, even as ATP levels remain high, hydrolysis of ATP to support physiological needs results in the generation and accumulation of hydrogen ions that reduces the pH of the muscle. The rate of pH decline closely reflects the accumulation of lactic acid, which serves as a marker of the rate of postmortem glycolysis [9]. As we shall see later, extreme variations in the rate of postmortem glycolysis are often associated with meat quality problems. The length of time associated with the delay phase is quite variable, depending on species, animal genetics, antemortem nutritional status, and management of the animal prior to slaughter, and possibly slaughter method. With red meats, this period may last up to 12 h, whereas with poultry, this period may be in the range of 30 min to 2 h [2].

When CrP is near depletion, the concentration of ATP begins to decline precipitously as utilization exceeds the regenerative capacity. Thus begins the rigor phase (Figure 16.15). As ATP levels fall, there is a corresponding increase in rigor development, measured by the increase in resistance of muscle to stretch. Depletion of ATP reduces the ability of the calcium pump protein to maintain sarcoplasmic Ca^{2+} concentrations in the submicromolar or resting muscle concentration range. Likewise, there is a gradual increase in the number of myosin crossbridges with actin that remain locked because of a lack of ATP to dissociate the two proteins, leading eventually to a maximal stiffening or inextensibility of the muscle. As might be expected, some degree of sarcomere shortening may take place during this period, the magnitude of which is closely related to meat toughness.

The final phase of conversion of muscle to meat is referred to as aging or rigor resolution. This period may last from a few days in poultry, pork, and lamb, to 2 weeks for beef. During the resolution phase, there is a gradual increase in muscle extensibility and tenderness. The basis for these favorable changes is largely the result of proteolytic disruption of the myofiber ultrastructure, most notably at the Z-discs that lose structural integrity rapidly as a function of time. Although development of tension during rigor has important implications in the eventual resolution of rigor, it is widely accepted that proteolysis is largely responsible for the disruption of muscle structure and increase in meat tenderness [71].

16.4.1 Postmortem Degradation of Muscle Proteins

It is recognized that as muscle undergoes conversion to meat, the tenderness of meat tends to increase as a function storage time postmortem. This tenderization may be at least partially the result of degradation of both the myofibrillar and cytoskeletal proteins in muscle [72].

Among the muscle proteases, calpain has been studied most intensively with regard to its role in postmortem muscle protein degradation. Calpain is a calcium-activated, cysteine-protease that is most active in the neutral pH range. Calpain is regulated by a variety of factors, including calcium, phospholipids, and calpastatin, a widely distributed calpain-specific protein inhibitor [73]. Muscle tissue primarily expresses three different calpains: the two ubiquitous calpains and calpain 3 [74]. Ubiquitous calpains include μ-calpain, which requires 5–50 μM Ca^{2+} for its half-activation, and *m*-calpain, which requires 0.25–1 mM Ca^{2+} for its half-activation. These two isoforms are ubiquitously expressed in tissues, suggesting their involvement in basic and essential cellular functions mediated by the Ca^{2+} signaling pathway. Calpain 3 (also called p94, or CAPN3) is a skeletal muscle-specific calpain isoform and it has a lower Ca^{2+} requirement for its activation. Ubiquitous calpains are composed of two subunits: a catalytic subunit of $M_r \sim 80,000$ and a regulatory subunit of $M_r \sim 30,000$. The regulatory subunits are homologous between the two isoforms, but the catalytic subunits are slightly different. Calpain 3 possesses the classical structure of a calpain except that it carries three unique sequences not found in any other calpains. Ubiquitous calpains tend to be concentrated in the Z-discs and treatment of myofibrils with calpains causes rapid and complete loss of the Z-discs. In skeletal muscle cell, calpain 3 binds specifically to certain regions of titin [75]; however, calpain 3 does not cut titin. As the Ca^{2+} concentration increases postmortem, calpains (mainly the ubiquitous calpains) are activated and initiate the degradation of muscle proteins such as troponin-T, titin, nebulin, C-protein, desmin, filamin, vinculin, and synemin [72]. Most of these proteins are either directly attached to (e.g., titin, nebulin), or closely associated with (e.g., filamin, desmin, synemin), or near (vinculin) the myofibrillar Z-discs. When Z-discs are almost completely disrupted, actin and myosin are passively released together with other proteins from the sarcomere and become substrates for other proteolytic enzymes.

Cathepsins are lysosomal proteases that are maximally active at acidic pH, a condition that prevails for the remaining postmortem period, particularly the aging period. At first, this suggests that cathepsin activity is likely to be more important than calpains for achieving the desired tenderization effects during aging. However, when proteinases are incubated with myofibrils, it is the activity of the calpains that closely mimics the proteolytic events in postmortem tenderization. Furthermore, postmortem tenderization is Ca^{2+}-mediated [76], a characteristic only associated with the calpain proteolytic system in skeletal muscle. Still, the importance of cathepsins in aging of meat cannot be overlooked. Their intracellular location and activity on numerous proteins in postmortem skeletal muscle make them logical candidates for postmortem proteolysis and tenderization [77].

The proteosome is a large ubiquitous ATP- and ubiquitin-dependent proteolytic system that may also be involved in myofibrillar protein degradation during aging of meat. The proteosome is able to degrade actin and myosin *in vitro* [78]; however, the proteasome is not able to degrade intact myofibrils. Muscle proteins such as actin and myosin are released from the sarcomere by a Ca^{2+}/calpain-dependent mechanism before they undergo ubiquitination and degradation by the proteasome. In animals overexpressing calpastatin, postmortem degradation by the proteasome is reduced, confirming the involvement of calpains [79]. Therefore, calpain may be the initiator of myofibrillar degradation and the proteasome may be responsible for proteolytic reactions that remove all myofibrillar fragments and hydrolyze them to amino acids.

16.5 NATURAL AND INDUCED POSTMORTEM BIOCHEMICAL CHANGES AFFECTING MEAT QUALITY

Consumer choices in meat purchases are strongly influenced by various product attributes including water-holding capacity, color, fat content, and tenderness of the meat. Meat cuts displaying abnormally light or dark color, or excessive loss of moisture within the package are more likely to be rejected by consumers, thereby downgrading the product value. Likewise, an unsatisfactory

experience by a consumer with an unusually tough cut of meat may result in subsequent product rejection.

The quality attributes of meat are influenced by multiple interacting factors including animal species, breed, genotype, nutritional status, preslaughter handling, and postmortem chilling, processing, and storage. Some specific examples of quality problems and their underlying molecular bases are described in the following sections.

Following aging of meat, the characteristics of the tissue differ substantially from that of live muscle. Postmortem metabolism has led to a decrease in pH from the physiological value of ~7.4 in muscle tissue, to an ultimate pH of ~5.5–5.9 in red meat and poultry. In addition, a degree of contraction has taken place in the tissue prior to the formation of rigor complex.

The consequences of reduced pH are simultaneously beneficial and detrimental to the value of the product. Clearly, the acidic pH of meat will retard microbial growth and thereby extend shelf life compared to the neutral pH of muscle. However, this advantage is offset by the economic loss to processors resulting from loss of water from the tissue as the pH becomes increasingly acidic. The isoelectric point of myosin (the dominant protein in muscle) is approximately 5.0; at this pH, the sum of positive and negative charges is zero, protein–protein interactions are maximal, and protein–water interactions are minimal. As a result, myofibrils shrink and lose much of their water-holding capacity. This loss of water during storage of fresh or cooked product (sometimes referred to as "purge") may be quite substantial, resulting in reduced value because the product bathed in its exudate is unattractive. Moreover, because meat is sold by weight, loss of water equates to loss of product weight and thus, decreased profitability. The product that has lost substantial water content will be perceived by the consumer as having both a reduced juiciness and tenderness. It must also be acknowledged that the watery exudate carries significant quantities of water-soluble vitamins, minerals, amino acids, and other nutrients. These nutrients in the exudate would be lost to the consumer. In addition to reduced water-holding capacity, visual defects may also be brought about by rapid postmortem glycolysis and a low ultimate pH being attained.

16.5.1 Pale, Soft, Exudative Meat

All muscle tissue undergoes a reduction in pH as a result of ATP hydrolysis during the conversion of muscle to meat. However, in aberrant cases, the rate of pH decline is unusually rapid such that most of the pH reduction takes place while the carcass temperature is high. For example, within 45 min postmortem, pig muscle pH is typically in the range of 6.5–6.7 while the temperature is approximately 37°C. In some carcasses, however, the pH may drop to less than 6.0 during the same time period. It is the latter combination of rapidly decreasing pH while the carcass temperature is still high that results in denaturation of some of the contractile proteins, with consequent loss of water-holding capacity, leading to the phenomenon known as "pale, soft, exudative (PSE) meat" [80]. Although these attributes of the fresh cuts are likely to lead to rejection by consumers, the reduced protein functionality of the PSE product in processed meats has also serious economic consequences for the processing industry.

The molecular basis for the PSE meat problem has been the subject of intense investigation over the past half-century. It is clear that antemortem stressors of the animal, such as heat, transportation, physical exercise, mixing of unfamiliar animals, and animal handling, are significant contributors to the problem of meat quality. The exact mechanism by which these stressors result in PSE meat is still unclear, but it is evident from a variety of studies that reduction of antemortem stress results in significant improvement in overall meat quality.

An animal's genotype may further increase its predisposition to an adverse response to stress. In the 1960s it was noted that subsets of pigs within various breeds were particularly susceptible to stressors. The term "porcine stress syndrome (PSS)" was coined to describe the inheritable muscle disorder of the pigs with low tolerance to the effects of stress [81]. These animals typically responded to stressors by developing a condition known as malignant hyperthermia, which is characterized by

severe muscle contracture, respiratory distress, rapid-onset of high fever, and eventually death. Animals with this disorder that did not succumb to the effects of stress were much more likely than normal animals to yield PSE meat.

Studies over the next 30 years eventually led to the identification of a mutation in the RyR that was responsible for PSS. The substitution of thymine for cytosine at nucleotide 1843 in the RyR coding sequence leads to substitution of cysteine for arginine at residue 615 [82]. This mutation leads to excessive calcium release from SR in the stressed animal, which, in turn, triggers severe muscle contracture and eventually malignant hyperthermia in the live pig. Excessive postmortem calcium release in the muscle of the PSS-susceptible pig triggers muscle contraction and associated anaerobic glycolysis with consequent hydrogen ion accumulation and heat production associated with the development of PSE pork.

The problem of PSE meat has classically been associated with pork; however, in the early 1990s, increasing incidence of PSE meat became apparent in the turkey processing industry. The striking similarity in the development of PSE pork and turkey led to the suggestion that a mutation in the RyR is responsible for the problem of PSE turkey [83]. As noted in Section 16.3.4.6, there is a significant difference in the excitation–contraction coupling mechanisms between mammals and birds. Thus, it is possible that if a mutation exists, it may be either in the RyR1 or RyR3 isoforms, or in both. To date, no mutations has been identified in either turkey RyR isoform. However, there are intriguing indications that various alternatively spliced RyR transcript variants can be expressed in turkey skeletal muscle that may alter the tendency to produce PSE meat [84].

Another genetic abnormality that can lead to PSE meat from pigs is the *Napole* (RN) gene. The mutation in this case is a substitution of glutamine for arginine at residue 200 of the γ-subunit of AMP-activated protein kinase [85]. This enzyme plays a variety of roles in muscle including activation of ATP-producing pathways and inhibition of ATP-consuming pathways as well as inactivation of glycogen synthase.

Pigs possessing the dominant RN− allele tend to have much higher glycogen content than pigs with the recessive rn+ allele. The rate of postmortem pH decline in muscle from pigs with the *Napole* gene tends to be normal. However, the high levels of glycogen tend to lead to an extended pH decline and consequently in a very low ultimate pH, resulting in PSE meat with poor protein functionality. In fact, although the water-holding capacity is reduced compared with normal pork, the protein functionality of RN– pork in processed meats is even lower than pork possessing the RyR abnormality. The fact that 65–80% of pork is consumed as processed meats demonstrates the importance of eliminating the *Napole* gene from pigs.

16.5.2 Dark, Firm, and Dry Meat

An occasional consequence of preslaughter stress is antemortem depletion of glycogen stores through stressors, exercise, or excessive fasting. This leads to a product that has the opposite characteristics of PSE meat, and is referred to as "dark, firm, and dry" or DFD meat [86]. The meat color may vary from slightly dark red to extremely dark or nearly black in contrast to the normal cherry red appearance of normal red meat. The problem is most notable in beef, but has also been reported in pork. The lack of adequate glycogen reserves results in early termination of glycolysis, so the ultimate pH remains relatively high (>6.0). The incidence of DFD meat tends to be seasonal, with higher incidence when animals are exposed to sustained periods of cold, damp weather as compared with summer.

Dark, firm, and dry meat has much higher water-holding capacity than normal because the pH is further from the isoelectric point of myosin (~5.0). However, this advantage is strongly offset by the susceptibility of the product to growth of microorganisms and the rejection by consumers because of the abnormal color.

The unusually dark color of this product is a product of the high postmortem pH that keeps the charge on the muscle proteins high, thereby maximizing separation of muscle myofibrils and

reducing light scattering. Active mitochondrial respiration at the higher pH also reduces the fraction of oxymyoglobin in the tissue.

16.5.3 Cold Shortening

In the early 1960s the New Zealand lamb processing industry began receiving complaints from importers in Europe and North America that the meat was excessively tough. This was in contrast to lamb consumed in New Zealand that was not considered to be tough, suggesting that processing and/or storage factors may have been playing a role in the meat toughness [87].

A series of studies from the New Zealand Meat Research Institute elegantly demonstrated the complex relationship between prerigor chilling and meat toughness (Figure 16.16). When prerigor muscles are excised from the bones to which they are attached, they undergo contraction. The extent of contraction depends on the type of muscle (red vs. white), amount of time postmortem, physiological state of the muscle, and temperature. At near-physiological temperatures, the degree of shortening is high. At lower temperatures, the extent of muscle contraction decreases progressively until a nadir is reached in the range of 10–20°C, temperatures at which minimal shortening takes place. If the same muscles are subjected prerigor to still lower temperatures, one notes a dramatic increase in the degree of shortening at temperatures below 10°C. The cold-induced muscle contraction is termed "cold-shortening."

Marsh and Leet [88] subsequently demonstrated the relationship between prerigor cold-shortening and toughness (Figure 16.17). The Warner–Bratzler shear test measures the amount of force required to cut through a fixed size of muscle tissue. Using this test, Marsh and Leet demonstrated that as the degree of shortening increased, the degree of toughness (measured by shearing force) increased, up to the point when muscle had contracted by about 40%. At this point, there is maximal overlap between the thin and thick filaments, and nearly all of the myosin heads are bound

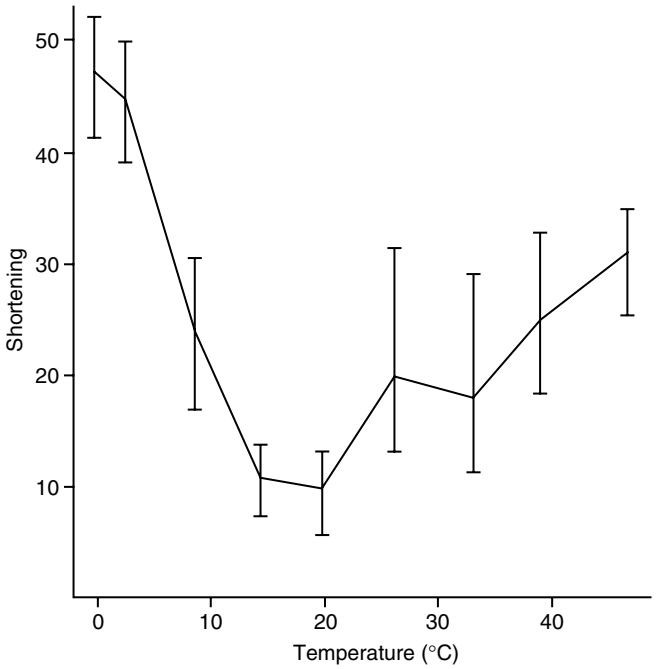


FIGURE 16.16 Shortening of excised prerigor beef muscle as a function of storage temperature. (Reprinted from Locker, R.H. and C.J. Hagyard (1963). *J Sci Food Agric* 14:787–793.)

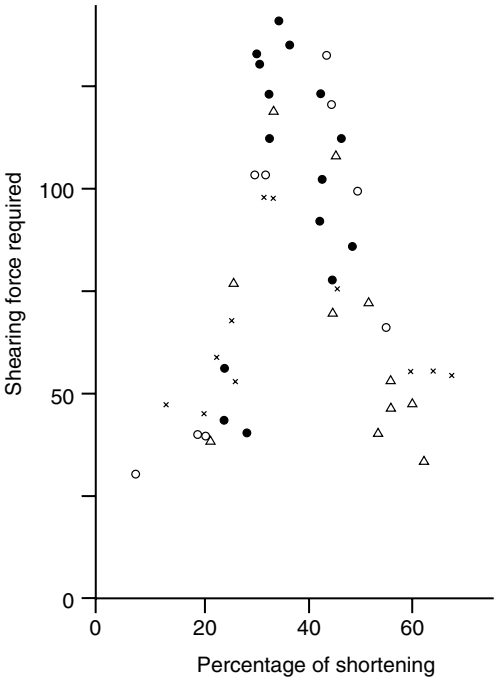


FIGURE 16.17 Meat toughness measured by shear force required to cut through a meat sample as a function of sarcomere shortening. Muscle shortening was induced by storage at various temperatures as well as by thaw rigor. (Reprinted from Marsh, B.B. and N.G. Leet (1966). *Nature* 211:635–636.)

to actin filaments. Beyond this point, additional contraction may occur resulting in a decrease in toughness. The explanation for this observation is that under some circumstances such as lack of tension, muscle contraction may continue to the point that the thick filaments puncture the Z-disc, causing extensive damage to the muscle ultrastructure and thus leading to an increase in tenderness [89]. On the basis of these observations, substantial modifications were made to the postslaughter chilling of lamb carcasses to reduce prerigor chilling rate. Subsequent studies in the United States and Europe have validated the significance of cold-shortening in the red meat industry and led to practices to minimize this problem.

The mechanism underlying the development of cold-shortening is still not completely clear, but the following factors clearly play a role. Muscle must be in the prerigor state for contraction to occur; only during this period are there adequate levels of ATP to provide the energy for contraction as well as for dissociating myosin heads from actin for a subsequent contraction cycle. The temperature must be lower than 10°C, and the closer to 0°C, the greater the degree of shortening. Finally, the muscles most susceptible to cold-shortening are those with the highest percentage of red fibers, for example, beef and lamb or sheep muscles. Cold-shortening occurs to a lesser extent in pork because most pig muscles have higher percentage of white muscle fibers. Relatively little cold-shortening occurs in poultry muscle.

Various lines of evidence suggest that altered postmortem calcium regulation and temperature-dependent changes in enzyme activities are responsible for the cold-shortening phenomenon. As the temperature of the muscle goes below 10°C, anoxic mitochondria and SR lose their ability to retain Ca^{2+} ions. The calcium pump protein would normally keep sarcoplasmic Ca^{2+} concentrations at resting muscle levels. However, at the lower temperatures, the rate of SR reaccumulation of Ca^{2+} is suppressed; thus, sarcoplasmic Ca^{2+} concentrations increase, thereby triggering muscle contraction. Red muscle fibers are more likely to exhibit cold-shortening. They rely primarily on oxidative

metabolism to support energy requirements; thus, they have a higher amount of mitochondria than white fibers. Moreover, red fibers tend to have a less developed SR network, thereby reducing their ability to reaccumulate Ca^{2+} [86].

Strategies to prevent cold-shortening were initially built on the relationship between the rate at which the carcass was chilled and development of toughness. Keeping carcass temperatures in the range of 10–20°C until rigor mortis is established and prevents cold-shortening. However, this approach is in conflict with the desire of lowering carcass temperatures quickly to minimize microbial growth on the surface of the meat. In the 1970s, experiments with electrical stimulation of carcasses demonstrated that the time for development of rigor mortis could be dramatically shortened [90]. This procedure is described in Section 16.5.5, and has now become standard industry practice for most beef and lamb slaughter operations.

16.5.4 Thaw Rigor

A phenomenon closely related to cold-shortening is the severe muscle contracture that takes place upon thawing muscle that had been frozen while still in the prerigor condition. Thaw rigor was first described by Sharp and Marsh [91] who noted that thawing of prerigor whale muscle resulted in shortening of the muscle by as much as 60%, and was accompanied by substantial loss of water-holding capacity. Thaw rigor likely results from structural damage to the SL and SR membranes from ice crystal formation. The loss of membrane integrity results in Ca^{2+} influx into the sarcoplasm triggering muscle contraction because ATP levels are still sufficiently high for shortening to occur. Likewise, membrane damage results in excessive drip loss from the muscle fibers upon thawing. The same approaches to preventing cold-shortening, electrical stimulation or holding muscle on the carcass at temperatures above freezing until the onset of rigor mortis, will serve to prevent thaw rigor.

16.5.5 Electrical Stimulation

Electrical stimulation refers to the application of an alternating electrical current to carcasses of meat animals following slaughter. When applied to carcasses during the early postmortem period, electrical stimulation induces extensive muscle contraction and relaxation, which consequently accelerates the rate of muscle metabolism, ATP turnover, and rigor development. Depending on the voltage, frequency, and duration of the stimulus, this procedure reportedly improves the tenderness, flavor, color, quality grade, retail case life, and processing characteristics of meat and meat products [92].

Improvements in color and quality grade have been primarily responsible for the adoption of electrical stimulation by the processing industry in the United States. In other countries, the primary reason has been improvement in tenderness. Use of electrical stimulation was initially adopted in New Zealand, where the practice of rapid chilling and freezing of lamb carcasses for export led to cold-shortening, thaw-shortening, and increased meat toughness [90]. However, electrical stimulation is also effective in improving meat tenderness in the absence of cold-shortening. Pearson and Dutson [93] discussed mechanisms for tenderization. Lysosomal disruption with consequent release of endogenous proteases has been suggested as a mechanism, based on an increase in the free activity of lysosomal enzymes after electrical stimulation. Thus, increased proteolytic activity prior to and during the aging period could account for a portion of the increased tenderness. Another mechanism appears to be the physical disruption of myofiber integrity caused by the extensive contractions induced with electrical stimulation [94]. Zones of supercontraction (contracture bands) and excessive stretching of the myofilaments adjacent to contracture bands are evident in photomicrographs of meat from electrically stimulated carcasses. On the basis of the physical dimensions of thick and thin filaments, contractions beyond 40% of resting sarcomere length would result in the thick filaments penetrating Z-discs and interacting with thin filaments of adjacent sarcomeres [89]. Other areas must stretch and/or tear to accommodate these zones of supercontraction. An increase in tenderness

would ensue. Although conclusive support is still lacking for the case of physical disruption, the proposed mechanism is consistent with other instances of tenderization associated with excessive contraction.

The beneficial effects of electrical stimulation are thought to occur because of the acceleration in the postmortem conversion of muscle to meat. In essence, stimulated carcasses reach their maximum quality grade sooner, which allows the packer to either increase production at similar quality grade levels or increase quality grade levels at similar production [92]. Where quality grade influences the pricing system, either case is financially more favorable for the packer and accounts for the extensive use of electrical stimulation in the beef industry.

16.6 CHEMICAL CHANGES IN MEAT DURING PRESERVATION

The susceptibility of muscle tissue to microbial spoilage requires that meat be preserved by physical or chemical methods. Refrigeration and freezing provide the most effective means to retard microbial growth as well as to minimize deleterious chemical and biochemical processes in meat and meat products. Traditionally, fresh meat is preserved by salting and partial dehydration that raise the osmotic pressure and lower the water activity thereby suppressing the growth of microorganisms. Irradiation and high-pressure treatments are relatively new preservation interventions that are becoming acceptable in the meat industry. On the other hand, nontraditional packaging systems, such as modified atmosphere packaging, are gaining popularity to extend meat shelf life. All these preservation techniques affect not only the ecology of microorganisms on meat but also the chemical properties of meat and meat products.

16.6.1 Chilling and Refrigeration

In a typical packing plant, animal carcasses are rapidly chilled in a 2–5°C chill cooler to minimize microbial growth; for chicken broilers and fish, the carcasses are usually chilled by immersion in ice slurries. The time duration for a warm carcass to reach its final chilled temperature varies, depending on its size, thickness of subcutaneous fat, and the chilling methods. The chilling time can be as short as 1 h for an ice-chilled broiler carcass and up to 24 h for a 300-kg beef carcass. At some commercial packing plants, cold water showering and high-velocity air are used to facilitate the chilling process.

The rate of chilling affects the enzymatic reactions in postmortem muscle tissue, which in turn affects the quality of aged meat. The major biochemical changes occurring during the early stage of muscle to meat conversion—the pH fall (glycolysis), the depletion of ATP, and muscle contraction—are enzymic processes. As the carcasses are rapidly chilled, these biochemical changes are retarded due to the inhibition of enzyme activities. Lipid oxidation also proceeds more slowly at refrigeration temperatures due to the reduced activity of oxidative enzymes. Inhibition of lipid oxidation would preserve the fresh meat flavor and minimize myoglobin oxidation. However, exposing prerigor muscle to cold temperatures could have an adverse effect on meat tenderness due to increased muscle fiber contraction or shortening, notably for red meat. To minimize cold-shortening, it is imperative that muscle remain attached to the skeleton during chilling, especially in the early stage of postmortem storage. Because the activity of major endogenous proteases involved in meat tenderization during postmortem aging, for example, calpain and cathepsins, is greatly reduced at low temperatures, it is necessary that the carcass or meat be adequately aged to achieve an acceptable level of tenderness.

16.6.2 Freezing

Freezing is one of the most effective methods of preserving meat. When meat and meat products are stored at temperatures below −10°C, microbial growth and enzyme reactions are essentially

curtailed, and hence, quality loss is minimized. However, physical and chemical reactions can still occur in meat during freezing, storage, and subsequent thawing. Chemical changes in frozen meat during storage include discoloration and development of oxidative rancidity, which result from oxidation of myoglobin and unsaturated lipids, respectively, and texture hardening due to protein denaturation and aggregation. These adverse changes are influenced by the rate of freezing and thawing, the duration of frozen storage, fluctuations of the freezer temperature during storage, and the atmospheric condition of the frozen meat. In the case of processed meat, the ingredients added to meat (e.g., NaCl) and the specific processing procedures, such as grinding, chopping, emulsification, and restructuring, can influence the quality and shelf life of the frozen products. Antioxidants are often added to inhibit salt-induced oxidation in frozen meat products.

Freeze-induced protein denaturation, a main side effect of frozen meat, is attributed to physical damage resulting from the formation and accretion of ice crystals, and from chemical processes associated with dehydration and concentration of solutes in the muscle tissue. Freeze-induced protein denaturation is especially notable under slow freezing conditions. At a slow freezing rate, the exterior fluid of muscle cells cools more rapidly than the interior fluid, and when the supercooled extracellular fluid reaches a critical temperature, water separates from solutes and forms ice crystals. As crystallization proceeds, extracellular salt becomes more concentrated, creating an osmotic pressure gradient across the cell membrane. These processes can lead to protein denaturation and disruption of the cell membrane [95]. To prevent protein denaturation, cryoprotectants, such as polyphosphate and polyols (sorbitol, sucrose, polydextrin, etc.), can be incorporated into meat prior to freezing.

The rate of freezing is dictated by freezing methods employed, and follows the order of cryogenic freezing > blast-freezing > still-freezing. Cryogenic freezing, which uses condensed gases such as liquid nitrogen (−195°C) and solid carbon dioxide or dry ice (−98°C), rapidly chills meat to below 0°C and transforms liquid water into ice crystals in a matter of minutes. Blast-freezing air (e.g., −50°C) also enables a rapid heat transfer thereby crystallizing intramuscular water in a very efficient manner. On the other hand, still-freezing allows slow heat dissipation from meat, and can cause damage to muscle cell and proteins. In general, fast freezing promotes the formation of small ice crystals that are uniformly distributed inside and outside the muscle cells, while slow freezing favors the formation of large ice crystals that are much less in quantity and are prevalent extracellularly.

A relatively new freezing technology, known as "pressure-shift freezing," has been introduced as a potential meat quality preservation method. During pressure-shift freezing process, meat samples are chilled to subfreezing temperatures (e.g., −20°C) and will not freeze under a certain high pressure. When the pressure is suddenly released, instantaneous and homogeneous microcrystallization occurs throughout the muscle tissue. Meat processed with pressure-shift freezing reportedly has a minimally altered ultrastructure, reduced protein denaturation, and an improved product quality [96,97].

16.6.3 Pressurization

High-pressure treatment is potentially useful for meat preservation as well as for processing to improve product quality. Hydrostatic pressures ranging from 100 to 800 MPa have been used to destroy pathogenic microorganisms and inactivate spoilage enzymes in fresh meat prior to storage. Nonthermal, high-pressure processing is also used to process postpackaging ready-to-eat meats, such as deli slices, to eliminate potential contamination by *Listeria monocytogenes*. Because the compression energy is low (e.g., 19.2 kJ for 1 L of water under a 400 MPa pressure), covalent bonds are usually not affected. However, high-pressure treatment may disrupt electrostatic and hydrophobic interactions in proteins, thereby rendering them less stable. Because pressurization processes do not depend on additives or temperature, treated meat products will retain their flavor and taste.

Physical modifications in muscle tissue under high pressures include the decrease in the volume of the aqueous phase and a drop in pH. These changes are reversible upon pressure release. However, even a brief exposure to these temporary changes could permanently alter the protein structure

and its association with nonprotein compounds. Pressures at above 100 MPa can cause protein quaternary structure to dissociate into its subunits, monomeric structure to unfold, and can induce protein aggregation and gelation [98,99]. High-pressure treatment separates myosin heavy chains into one-headed monomers, which is followed by head-to-head interaction to form aggregates [100]. Pressure-induced changes in the hydration volume appear to play a major role in the unfolding, dissociation, aggregation, and gelation of muscle proteins. Pressure-treated muscle proteins, such as surimi, can spontaneously form a gel at mild temperatures. This results from increased exposures of hydrophobic side chain groups, allowing protein aggregation to readily occur.

High-pressure treatments of prerigor muscle increase the rate of glycolysis and fiber contraction, which is attributed to multiple factors, including the disruption of SR where calcium is normally stored and the loss of Ca-ATPase activity. The high cytosolic concentration of Ca^{2+} activates enzymes involved in glycolysis (e.g., phosphorylase kinase) and muscle contraction (e.g., myosin ATPase). When the applied pressure is sufficiently high (e.g., >400 MPa), discoloration occurs in raw meat as indicated by an increased L^* value and a decreased a^* value [98]. Indeed, exposures of raw meat to high pressures result in an increased metmyoglobin (brown) content at the expense of myoglobin (red) due to oxidation of heme iron and the denaturation of globin.

A pressure at above 150 MPa can induce extensive changes in sarcomere structure as well, for example, the disappearance of the M-line and H-zone and the loss of integrity of I-band filaments. Surprisingly, these structural changes do not seem to lead to meat tenderness improvement. This may be due to thickening of the Z-discs in costameres and the loss of protease activity [101]. On the other hand, collagen structure is not affected by high-pressure processing.

In high-pressure-treated beef, the level of μ-calpain is markedly reduced during aging [102]. Both μ-calpain and m-calpain are partly inactivated at 200 MPa and completely inactivated at 400 MPa. High-pressure-induced denaturation and the enhanced autolysis of calpains due to the high concentration of cytosolic Ca^{2+} released from SR are responsible for the negative effect. On the other hand, high-pressure application increases catheptic activity (B, D, L, H, and peptidases) due to the disruption of lysosomal membrane. The increased release of cathepsins from lysosomes apparently is sufficient to overcome the pressure-induced denaturation. Nevertheless, the enhanced catheptic activity is not able to compensate for the reduced tenderness resulting from the loss of calpain and structural changes in myofibrils. For this reason, softening of muscle tissue in fish during storage due to excessive proteolytic activities can be overcome by high-pressure treatments that inactivate endogenous proteases, especially lysosomal enzymes.

Extremely high pressures generated with pyrotechnic devices have been invented to tenderize meat. A particular example of such technologies is Hydrodyne® [103]. In this method, encapsulated fresh meat is placed in a sealed, water-filled container situated below the ground level. A small amount of explosive, consisting of a liquid and a solid, generates a shock wave that is in acoustic match with water in muscle. The shock wave produces an extraordinarily high pressure measuring about 680 atm or 10,000 psi at the contact surface with the meat. Meat exposed to such a high pressure exhibits remarkable tenderness improvements and requires less aging time to achieve desirable tenderness. The tenderizing effect is attributed to the disruption of the myofibrils, including the Z-discs. Because Hydrodyne also inactivates microorganisms, it has the additional benefits of preserving fresh meat and enhancing meat safety.

16.6.4 Irradiation

Irradiation as a means to inactivate pathogenic microorganisms has gained acceptance in the meat industry. There are two types of radiations: ionizing radiation and nonionizing radiation. In nonionizing radiation, such as microwave and infrared frequencies, the energy of radiation is not high enough to cause atoms to ionize. Instead, it relies on the heat it generates to destroy microorganisms and, hence, is suitable for heat-processed meat products. In ionizing radiation, a radiation generated by high-speed electrons or radioactive isotopes (γ-radiation) strike atoms to produce ions, and

destruction of microorganisms is therefore more effective. The permissible dosages of irradiation are 1.5–3.0 kilogray (kGy) for poultry and 7.0 kGy for beef.

γ-Irradiation is a proven method of radiation for fresh or raw meat. Although it is effective in reducing microbial contamination, adverse chemical changes to muscle tissue due to radiolysis do occur. For example, γ-irradiation of fresh meat can produce superoxide and hydroxyl radicals. These primary radicals are highly reactive and can react with muscle lipids and proteins to generate secondary radicals and lipid and protein degradation products. Differing from small radicals, which are short-lived in the aqueous environment of meat, protein radicals can be relatively long-lived and can cross-link with one another causing the muscle tissue to harden. On the other hand, degradation of unsaturated lipids in muscle following γ-irradiation leads to the production of various hydrocarbons, particularly alkenes and carbonyl compounds, which contribute to off-flavor of treated products. Volatile sulfur compounds are also produced by γ-irradiation due to radiolytic degradation of side chains of methionine and cysteine residues, and they are the main off-odor volatiles produced in irradiated, vacuum-packaged red meat and poultry [104].

Discoloration is another major consequence of γ-irradiation. Irradiated meat can develop unattractive greenish or brownish gray colors, which appear to be caused by the breakdown of the porphyrin structure of the heme or the formation of sulfmyoglobin [105]. For light-colored meat, such as poultry breast, an intense pink color can form that has been attributed to the formation of a carbon monoxide–myoglobin complex [106]. Because chemical changes caused by γ-irradiation are usually radical-driven processes, the use of vacuum-packaging or incorporation of proper water- and lipid-soluble antioxidants can minimize the negative impact on muscle food quality.

16.7 CHEMISTRY OF PROCESSED MEATS

Processing of meat refers to the application of physical, chemical, and thermal treatments of muscle tissue to increase the product variety, to offer convenience, and to extend meat shelf life. It involves extensive modifications of the physicochemical properties of fresh meat. Processed meats may be separated into three main categories: (1) those in which the structural characteristics of muscle are minimally altered, for example, cured ham and bacon, and corned beef; (2) those with moderately altered muscle structure, for example, sectioned and then restructured roasts and steaks; and (3) those that are extensively comminuted and then reformed, for example, sausage, frankfurters, and many luncheon meats. The chemical changes in the muscle tissue depend on the specific modification procedures and ingredients employed. For example, the development of a stable, pinkish red color in cured ham is due to the chemical reaction of nitric oxide with myoglobin; the formation of stable, fat globules in emulsion-type products is largely attributed to protein–lipid interaction at the water–oil interface; and the adhesiveness and smooth texture of boneless turkey ham result from interaction and gelation of myofibrillar proteins extracted by salt and phosphate.

16.7.1 Curing

The term "curing" refers to the treatment of fresh meat with salt and nitrite (or nitrate) for the purpose of preservation and obtaining desirable color and flavor. The origin of the curing technology is lost in antiquity, but is generally believed to be around 3000 BC. Cured meats have a characteristic pinkish color and a distinct aroma. They include traditional products such as ham, bacon, and summer sausage, and a variety of ready-to-eat products available in the deli or refrigeration section at retail outlets, for example, restructured and sliced turkey ham and deli-type bologna.

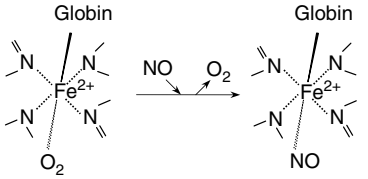
Sodium chloride (NaCl) is the common salt used in cured meats. The main functions of salt, other than imparting flavor, are to extract myofibrillar proteins and to increase osmotic pressure, thus inhibiting bacterial growth and subsequent product spoilage. Although salt is an indispensable ingredient in cured products, the actual curing agent is nitrite (NO_2^-) or nitrate (NO_3^-). Nitrate was

originally approved for color fixation in cured meats, but now it has largely been replaced by nitrite, because the latter is the immediate precursor of nitric oxide (NO), the ultimate curing compound. Nitrate is now restricted to dry-cured products, such as country-cured hams and dry sausages. In making these products, nitrate is slowly converted to nitrite by microorganisms or by reducing compounds, allowing slow curing reactions that presumably produce more desirable flavors and a stable color.

Nitrite is a multifunctional chemical. It induces and stabilizes the pinkish color of lean meat, contributes to the characteristic flavor of cured meat, inhibits the growth of spoilage and pathogenic microorganisms (particularly *Clostridium botulinum*), and retards development of oxidative rancidity. The pinkish red color characteristic of cooked, cured meats results from the reaction of the myoglobin heme with nitric oxide forming the nitrosylmyoglobin pigment. Nitric oxide is derived from nitrite in the presence of reducing compounds such as erythorbic acid. Part of nitrite dissolved in water can form nitrous acid (HNO_2). Under reducing conditions, nitrous acid decomposes to nitric oxide. When nitric oxide binds to the heme iron, it changes the electron distribution in the heme structure, thereby producing a pinkish color. Upon heating, nitrosylmyoglobin is converted to nitrosylhemochromogen, which is more stable due to globin denaturation.

$$NaNO_2 + H_2O \rightarrow HNO_2 + NaOH$$

$$3\,HNO_2 \rightarrow HNO_3 + 2\,NO + H_2O$$



Reducing compounds are added in meat curing mixtures to hasten color development via converting nitrite to nitric oxide, and ferric ion of the heme to ferrous ion. The most commonly used reducing compound is sodium erythorbate (an isomer of ascorbate). Muscle itself also contains endogenous reductants and enzymatic reducing activity, for example, cytochromes, quinines, and NADH, but the reducing power of these factors is relatively small. In addition to reducing metmyoglobin (Fe^{3+}) to myoglobin (Fe^{2+}), and nitrite to nitric oxide, erythorbate also serves as an antioxidant to stabilize both color and flavor, and to decrease the formation of nitrosamines. Phosphates, such as sodium pyrophosphate, tripolyphosphate, and hexametaphosphate, are other curing adjuncts. Phosphates do not directly enter the curing reactions but they function to increase water-holding capacity of muscle and contribute to oxidative stability by chelating pro-oxidative metal ions.

16.7.2 Hydration and Water Retention

As discussed in previous sections, water accounts for 70–80% of the weight in fresh meat. In injected or pumped meat, the water content can exceed 85%. The amount of moisture present in cooked meat determines the product juiciness and influences its tenderness. Water in meat is either bound or in a free form. Bound water is tightly associated with proteins through hydrogen bonds, which is influenced by the surface charge and polarity of protein. Free water is held via capillary forces in different compartments of the muscle tissue, for example, in the spaces between myofilaments, between myofibrils, and outside the fibers. This form of water makes up the bulk of the water in meat (70–90%). In comminuted meats, a large portion of water is also retained via entrapment in the matrix of myofibrillar protein gels. Denaturing conditions, such as frozen storage, oxidation, and rapid acid accumulation postmortem while the muscle temperature remains high, leads to reduced water binding in meat. An example of meat with poor water-binding ability is PSE pork and turkey, which has been discussed in previous sections.

The ability to bind, immobilize, and retain indigenous as well as exogenous water in processed meat is largely attributed to myofibrillar proteins, which are influenced by meat ingredients. High concentrations of monovalent salt (NaCl or KCl) solutions, that is, brine, are commonly incorporated into meat through marination or injection. Hydration and retention of added water are made possible through the NaCl-induced myofibril expansion owing to increased electrostatic repulsion, and thus, transverse swelling [107]. A variety of phosphate compounds, including sodium pyrophosphate, sodium tripolyphosphate, and sodium hexametaphosphate, are used in conjunction with salt to further improve the moisture-retention capability of meat. Injected fresh meat usually contains both salt (0.5–2.0%) and phosphate (0.25–0.40%). When an alkaline phosphate is used, it confers an additional benefit by raising the meat pH from around 5.5–5.6 (which is close to the isoelectric point of actomyosin) to 5.8–6.0 where myosin and most other muscle proteins will bind water more strongly due to increased net charges. A pH elevation would also allow interfilamental spaces to further expand via electrostatic repulsions for additional water to be immobilized.

The mechanism of NaCl- and phosphate-induced meat hydration extends beyond their simple electrostatic repulsion effect. In addition to increasing charge repulsions between adjacent myofilaments, high concentrations of NaCl (e.g., >2.5%) are able to dissociate myosin filaments, creating a bulky polypeptide matrix for moisture retention [108]. Furthermore, at elevated NaCl concentrations, the isoelectric point of myosin shifts to a lower pH due to screening of positive charges ($-NH_3^+$) in proteins by Cl^-. As a result, myosin (or actomyosin) within the normal pH range of meat will carry more surface charges (Figure 16.18). The increased interpeptide electrostatic repulsion enables a stronger protein–water interaction and a greater water-retaining capacity of meat. On the other hand, low concentrations of pyrophosphate and tripolyphosphate (<0.5% or 5–15 mM) are capable of dissociating the actomyosin complex. In the presence of magnesium, the dissociation effect of pyrophosphate is very similar to that exerted by ATP. The detachment of actin from myosin filaments allows water to more readily diffuse into the interfilamental spaces. The dissociation also improves meat tenderness. Significant muscle fiber swelling occurs as the NaCl concentration is raised from 0.1 to about 0.6 M in the absence of phosphate, or to 0.4 M in the presence of phosphate [109]. The extent of swelling and hydration continues until 1.0 M NaCl (~4.0% of muscle weight) is incorporated where the swollen fiber will start to shrink due a salt-out effect.

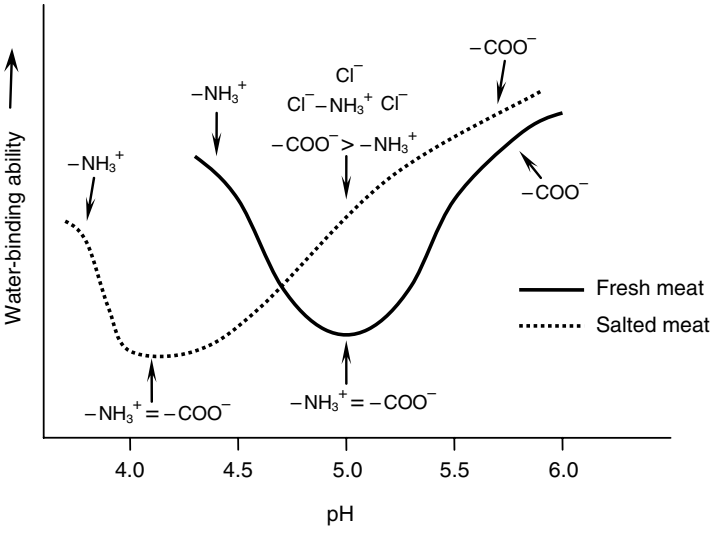


FIGURE 16.18 Schematic representation of the relationship between pH and water binding by proteins in fresh and salted meat.

Hydration of salt- and phosphate-treated meat is accompanied by partial extraction of myofibrillar proteins. Selective removal of proteins from the myofibril backbone may be necessary for the transverse expansion of the protein filaments. Phase contrast microscopy shows that myofibril "swelling" and removal of proteins from the thick filaments (myosin) in the 0.6–1.0 M NaCl solution occur concurrently [107]. The addition of 10 mM pyrophosphate or tripolyphosphate greatly facilitates the hydration process, and causes myosin to be extracted from the ends of the A-band where myosin cross-links with actin. Moreover, the extraction of transverse structural polypeptides, for example, M-protein, X-protein, and C-protein, by the presence of salt and phosphate, seems to promote loosening of myofibril lattices, thereby allowing water pickup by muscle fibers [110,111].

16.7.3 Formation of Protein Gel Matrix

Gelation of proteins is a physicochemical process involved in restructured and comminuted meat products. The gel formation is not only responsible for adhesion of meat pieces and particles, but also plays an important role in water, flavor, and fat binding in cooked products. Gelation in heated muscle foods takes place as a three-step sequential process. The initial unfolding (denaturation) of individual protein molecules is followed by their aggregation, largely through hydrophobic interactions; and at the final step, small protein aggregates or oligomers are cross-linked to form fine strands that eventually lead to a continuous viscoelastic network [112]:

$$\chi P_{\mathrm{N}} \xrightarrow[\text{Denaturation}]{\text{Heating}} \chi P_{\mathrm{D}} \xrightarrow[\text{(aggregation)}]{\text{Heating}} (P_{\mathrm{D}})_{\phi}, (P_{\mathrm{D}})_{\psi}, \cdots \xrightarrow[\text{(cross-linking)}]{\text{Heating/cooling}} (P_{\mathrm{D}})_{\phi} - (P_{\mathrm{D}})_{\psi} - \cdots,$$

where χ is the total number of protein molecules, ϕ and ψ ($\phi + \psi + \cdots = \chi$) are the number of molecules that are aggregated at certain point of the gelation process, P_{N} is native protein, and P_{D} is denatured protein. Examples of gel-type products are bolognas, frankfurters, and various luncheon meats made from comminuted muscle. Because of its adhesion ability, the gel formed at the junction of meat chunks in restructured products (e.g., boneless ham and turkey rolls) is largely responsible for the product integrity and sliceability.

Sarcoplasmic and connective tissue (stromal) proteins play only a minor role in the overall gelation phenomenon in processed meat. Most sarcoplasmic proteins are readily coagulated when salted meat is cooked to 40–60°C and they do not form an ordered, functional gel structure. Partially hydrolyzed collagen (gelatin) is the best-known gelling protein and its gelation is relatively insensitive to ionic strength. Gelatin forms reversible, cold-set gels, which are stabilized by hydrogen bonds. However, dissociation and degradation of collagen into soluble gelatin (the gelling component) requires moist, prolonged heating, a condition that is not commonly employed in producing muscle foods. On the other hand, myofibrillar proteins as a whole are superior gelling proteins, playing a vital role in producing desirable textural characteristics in processed muscle foods. In particular, myosin (prerigor) or actomyosin (postrigor) accounts for most of the gel-forming capacity of the myofibril protein system [113].

In order to form a gel, myofibrillar proteins must be extracted first, and this is usually initiated by mixing meat with salt (NaCl or KCl) and phosphates. The gelling properties of myofibrillar proteins are influenced by the proteins' structure and size, their concentrations, the source or type of meat, and the various processing conditions such as pH, ionic strength, and heating rate. Thus, myosin, which has a large length-to-diameter ratio (approximately 100 nm in length and 1.5–2 nm in diameter), can form a highly viscoelastic gel, whereas actin, which is a globular protein of about one-tenth of the myosin size, is a poor candidate for gelation [114], although it may reinforce myosin gels at a myosin-to-actin ratio (w/w) of about 24 [115]. Myofibrillar proteins from muscle white (FG) fibers form more rigid gels than those from red (SO) fibers, and this is attributed to the different physicochemical characteristics existing between myosin isoforms [116]. This explains why chicken pectoralis major, which consists exclusively of white fibers, and its myosin or mixed myofibrillar

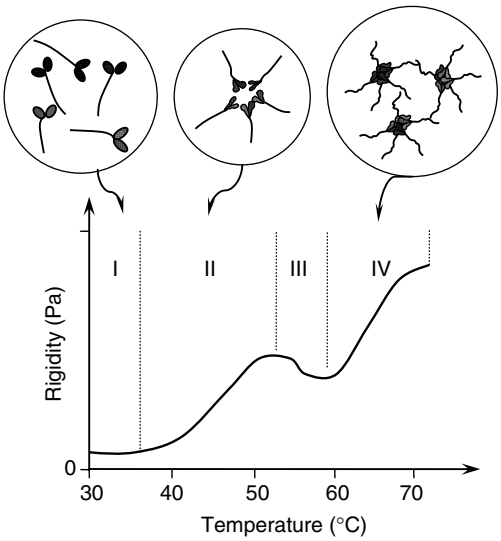


FIGURE 16.19 Schematic representation of heat-induced gelation of myosin in a 0.6 M NaCl, pH 6.0 solution. The four temperature zones show, respectively, (I) no change in myosin, (II) head–head association, (III) structural rearrangement of myosin aggregates owing to unfolding of light meromyosin, and (IV) cross-linking of myosin agglomerates via tail–tail association.

proteins, forms stronger gels than chicken gastrocnemius (preponderantly red fibers) or its proteins under equal meat processing conditions. Another unique property of myofibrillar proteins is that they tend to form the strongest gel at pH around 6.0, although the exact pH optima vary slightly depending on muscle types and animal species.

The mechanism of heat-induced myofibrillar protein gelation is largely accounted for by myosin, the major gelling component in the salt extract of processed meat. Under a typical meat processing condition (pH 6.0, 0.6 M, or 2.5% NaCl), the gelation begins with unfolding of S-1 region of HMM when the protein sol is heated to about 35°C, leading to hydrophobic association through head–head interactions (Figure 16.19). The oligomers then coalesce at about 48°C and form intermolecular disulfide bonds, producing a somewhat elastic characteristic. When the temperature approaches 50–60°C, conformational changes in LMM (rod) occur, creating an open structure that exposes hydrophobic regions and specific side chain groups. The structural change results in a temporary decline in the elastic characteristic of the semigel. For actomyosin, the drop in the gel rigidity in this intermediate temperature region is also related to the detachment of actin. However, the ensuing association of LMM via tail–tail interactions upon further heating leads to the formation of permanent strands and filamentous gel networks with high elasticity and water-binding capacity that are stabilized by disulfide bonds.

Microbial transglutaminase, an enzyme that catalyzes acyl transfer reactions thereby cross-linking proteins through the glutamine–lysine bridge, has a remarkable effect on myofibrillar protein gelation. Incorporation of this enzyme into the gelling solution results in as much as ten-fold increases in myofibril gel strength, and hence, appears to be an excellent food ingredient for use in meat and surimi processing where meat binding is of main importance [117,118].

16.7.4 Fat Immobilization and Stabilization

Fat in processed meat, notably in emulsified products, is immobilized and stabilized by the formation of protein interfacial membrane and protein matrices. During comminution or emulsification, large fat particles or the adipose tissue are broken down to fine granules through shear. As the small fat

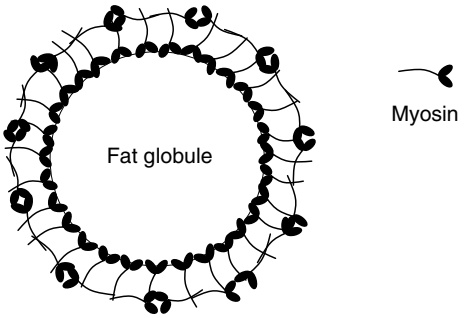


FIGURE 16.20 Schematic representation of a meat emulsion fat globule depicting a myosin monolayer.

globules are formed, they are coated by proteins, which are amphoteric in nature, that is, possessing both hydrophobic and hydrophilic groups. Specifically, the nonpolar groups are imbedded in fat (hydrophobic) while the polar groups extend into the aqueous phase, forming an interfacial film that separates the two immiscible phases (lipid and water). The adsorption of protein on the surface of fat globules decreases the interfacial energy, and denaturation of the adsorbed protein leads to the formation of a protein gel matrix; both these events enhance the emulsion stability. The relative emulsifying activity of muscle proteins follows the order of myosin > actomyosin > sarcoplasmic proteins > actin [119]. The excellent emulsifying capability of myosin is attributed to its unique structure (high length-to-diameter ratio) as well as to its bipolar nature (hydrophobic head and hydrophilic tail). A presumptive monolayer interfacial film, formed predominantly by myosin, is presented in Figure 16.20.

Fat globules in a well-comminuted meat, commonly referred to as "batter," are uniformly distributed in a continuous, yet, complex aqueous phase, which comprises salt-soluble proteins, segments of fibers, myofibrils, connective tissue fibers, collagen fragments, and various ingredients suspended in water. The two emulsion stabilization mechanisms—formation of a protein coating on the surface of fat particles to reduce the interfacial tension and immobilization of fat particles in protein matrices largely through physical entrapment—are applicable to meat batters. The coating, that is, protein membrane that surrounds fat globules, is not homogeneous but is multilayered in nature. Three distinct layers of proteinaceous structure have been observed in thick interfacial protein film of meat batters [120]. They are described as a thin, internal layer coating the surface of the fat globule, probably resulting from depositing proteins onto the myosin or actomyosin monomolecular layer (Figure 16.20). This innermost layer is bound through a diffuse region to another layer of similar density. This second layer is bound to a very thick, diffuse protein coat, forming a stable protein membrane structure.

Similarly, stabilization of fat by protein gel matrix is a complex physicochemical process because the gelling solution, referred to as protein "sol," is not simply proteins suspended in the aqueous solution. Rather, the sol represents a heterogeneous matrix consisting of soluble proteins with some insoluble, hydrated myofibril or fiber fragments suspended in it. Often, nonmeat ingredients (soy proteins, starch, seasonings, etc.) are also present. Hence, the gel formed after cooking may be considered as a composite system where aggregates formed from extracted myofibrillar proteins, fragments of myofibrils, and protein-coated fat globules interact, leading to an interwoven network. Some of the insoluble fibrils may entangle with the gel scaffold, whereas the fat globules may act as fillers in the void spaces of the gel matrix, thereby reinforcing the gel. The protein matrix gel is stabilized by a combination of forces, including hydrophobic and electrostatic interactions, hydrogen bonds, van der Waal's interactions, and covalent bonds, mostly through disulfide linkage. Covalent bonds, such as disulfide linkages, appear to be minor except when oxidation is involved.

The physicochemical and rheological properties of the fat globule membrane and the continuous protein matrices are the determinants of emulsion stability, and they are influenced by many factors,

including pH, viscosity of the aqueous phase, time and temperature of chopping or emulsifying, and the lean meat-to-fat ratio. The composition and properties of both the fat globule membrane and the continuous aqueous phase can be modified by means of ingredients and additives to obtain high-emulsion stability against coalescence at high cooking temperatures. Hydrolyzed soy, gluten, and whey proteins may help stabilize the emulsion system by reinforcing the interfacial membrane and the protein matrix. Treatment of emulsifying proteins with microbial transglutaminase prior to emulsification also enhances the emulsion stability and, additionally, enables the amorphous emulsion to form a stable gel matrix system upon heating [121]. It appears that the enzyme effects by means of promoting the interaction and cross-linking of membranes of fat particles as well as the interaction of the fat globules with segments of the protein network in the gel matrix.

16.7.5 Meat Restructuring

Restructured meats are fabricated and then reshaped products, and they include many formed raw steaks, chops, and roasts that resemble the intact products in texture. They also include a variety of cooked, ready-to-eat meats. Most cooked, ready-to-consume luncheon meats more or less fall into the restructured meat category. Restructured meats can be classified into three main groups, based on the specific method used and meat particle size reduction, that is, the extent of comminution: sectioned (entire muscle or muscle groups) and formed; chunked (coarse meat particles) and formed; and flaked (flakes from frozen meat) and formed.

Regardless of the method of reducing meat particle size, the single most critical factor affecting the product manufacture is the application and the action of salt. Salt (NaCl) is required to extract myofibrillar proteins, particularly myosin and actomyosin. The protein extract is a highly viscous, tacky exudate that provides adhesion between meat particles. For the bind to be effective, however, the protein extract must be converted to a viscoelastic, semisolid matrix, that is, a gel or semigel. This is made possible by heat when the formed meat is cooked. Extraction of salt-soluble myofibrillar proteins is accomplished by mixing meat with at least 0.5 M salt in a mechanical mixer, such as a massager and a tumbler. Tumbling relies on gravitational impact and abrasion from dropping meat pieces (previously tossed) against other meat pieces to extract myofibrillar proteins, while massaging relies mainly on abrasion between meat particles, and between meat and the rotating paddles of the massager to extract proteins. Polyphosphate is also used in conjunction with NaCl to improve protein extraction and moisture retention. Other ingredients, that is, seasonings, nonfat dry milk, whey protein concentrate, soy flour, and nitrite, may also be blended into restructured meat to accentuate the flavor and to enhance binding strength, texture, sliceability, and appearance.

Restructured fresh beef steaks and pork chops by the use of microbial transglutaminase have been successfully manufactured [122]. The enzyme forms covalent cross-links between glutamine and lysine side chains thereby bonding meat particles together. Meat products restructured without heating offer great flexibility and portion control, and can be marketed in specific shapes and sizes.

16.7.6 Chemistry of Surimi

Surimi is a crude myofibrillar protein concentrate prepared by washing minced, mechanically deboned fish muscle to remove sarcoplasmic constituents and fat, followed by mixing with cryoprotectants (usually polyols) to prevent protein denaturation during frozen storage. Surimi is an "intermediate" product because it is further processed to make various kamaboko (fish cakes) and seafood analogs, such as imitation crab meat and lobster, or utilized as a functional ingredient in other products.

A main quality concern with many of the fish species used for surimi is that they contain significant proteolytic activity that is detrimental to the texture of surimi-based products. For example, cathepsins B, L, and an L-like protease are difficult to be completely removed by the washing process. These endogenous proteases exhibit high activities in the 45–55°C temperature range, and thus,

can impair the textural properties of surimi-based cooked seafood analogs by degrading actomyosin [123]. Dry beef plasma protein, egg white, and potato extract are capable of preventing surimi gel softening and, hence, are blended into surimi before cooking is initiated. Small peptides (enzyme inhibitors) present in these additives may compete with fish muscle proteins as substrates for the proteases. Because the majority of the catheptic enzyme superfamily are cysteine proteases, cystatin (a general cathepsin inhibitor) is also used to prevent weakening of the surimi gel during cooking. This inhibitor can be efficiently produced using recombinant technology [124].

An alternative approach to the traditional surimi preparation method is acid or alkaline solubilization. Unlike the traditional surimi process, which employs repeated washing of the minced fish muscle tissue to concentrate myofibrillar protein by removing fat and sarcoplasmic constituents, the solubilization method involves either acid (pH 2.5–3.5) or alkaline (pH 9–10) treatment to solubilize both myofibrillar and sarcoplasmic proteins from homogenized muscle tissue. The soluble proteins are subsequently recovered by isoelectric precipitation (pH 5.0–5.5) [125]. This technique has the main advantage of high product yields (protein recovery >90% compared with 55%–65% from the traditional washing method). The acid solubilization and isoelectric precipitation method is particularly suitable for dark-muscle and fatty fish.

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17 Postharvest Physiology of Edible Plant Tissues

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CONTENTS

17.1 INTRODUCTION

17.1.1 Postharvest Deterioration and Losses of Plant Foods

Plant tissues, through the agency of photosynthesis, are ultimately the supplier of all food for humans. An estimated 150 billion tons of carbon are fixed annually by more than 300,000 taxonomically distinct plant species. Worldwide, plant tissues directly provide about 70% of the protein consumed by humans. In affluent countries, like the United States, about 30% of dietary protein comes directly from plants. Cereal grain contains 10,000–15,000 kJ/kg, about 10–20 times more energy per unit weight

TABLE 17.1
INQ for Seven Major Nutrients in Selected Plant- and Animal-Derived Food Products

Food	INQ > 1, Number of Nutrients	Total INQ (7 Nutrients)
Spinach	7	185
Broccoli	7	180
Lettuce	7	148
Orange	7	63
Apricot	7	45
Apple	2	8
Beans, navy	5	14
Potatoes	4	16
Whole wheat bread	5	8
Whole milk	4	17
Ground beef	4	13
Eggs	4	16
Tuna	3	17

Source: Data from Snook, J. T. 1984. *Nutrition*, Prentice-Hall, Englewood Cliffs, NJ, p. 521.

than most succulent fruits and vegetables. They are estimated to directly provide 50% of human calories in the world and 70–80% of all food calories consumed in China and India. Horticultural products are an important source of other dietary nutrients, as shown by the relatively high "index of nutritional quality" (INQ, defined as the ratio of the percentage of nutrient need provided to the percentage of caloric need provided) of several succulent crops [60]; the sum of INQ values for seven major nutrients is about ten times greater for spinach, broccoli, or lettuce than it is for whole milk, egg, or meat (Table 17.1).

Horticultural products are an important source of other dietary nutrients (vitamins and minerals) and non-nutrients (e.g., antioxidants) that are important components of a healthy diet. Horticultural products also generally have a lower caloric density than animal products so that the INQ is generally very high.

Although thousands of crops fit into this dietary picture, some 100–200 species are of major importance in world trade (Table 17.2). The major cultivated species produced in the world include sugarcane, wheat, maize, rice, potato, sugar beet, barley, cassava, sweetpotato, and soybean. These crops directly or indirectly provide an estimated 80% of human dietary energy and protein.

In the production of food crops, the main goal is to provide the consumer with a high-quality product they will buy. Tremendous resources are invested (i.e., to grow, harvest, pack, transport, market, etc.) in a crop that is grown, but it becomes wasted when it is unmarketable because of harvest and postharvest injuries and deterioration. Worldwide, postharvest deterioration accounts for about 40% of the total losses in potential food production [64]. Postharvest losses can come from improper environmental conditions (temperature, relative humidity (RH), atmospheric composition, light, etc.), pathogens (fungi, bacteria, viruses, etc.), physical damage, animals (rodents, birds, etc.), physiological disorders, or contamination.

The harvested plant commodities can be divided into two categories: cereal and oilseed crops, and perishable horticultural crops. Cereal and oilseed crops are generally dormant plant parts, have low moisture contents (10–20%), are small, have low respiration and heat production rates, are hard in texture, and have a shelf life of more than a year. The postharvest losses for these crops are usually

TABLE 17.2
Some Families in the Plant Kingdom That Are Important Sources of Human Food

Family	Examples	Characteristics
Gramineae	Grass family—cereals, sugarcane, bamboo	Directly and indirectly provide about 75% of food protein and energy; energy dense with 10,000–15,000 kJ/kg
Leguminosae	Legume family—peas, lentils, soybean, peanuts	Legume seeds average twice as much protein as cereals; soybeans are single largest cash crop in United States
Palmaceae	Palm family—coconut, date, palm	Source of oils
Rosaceae	Rose family—many fruits, e.g., apple, pear, peach, apricot, plum, cherry, raspberry, strawberry, rose hips	Includes several fruit structures, e.g., pome fruits, stone fruits, and aggregate fruits; develop distinctive aromatic flavors and sweet taste during ripening
Curcurbitaceae	Melon family—squash, cantaloupe, watermelon, cucumber, pumpkin	History of cultivation is >9000 years; fruit do not tend to sweeten as they ripen
Cruciferae	Mustard family—cabbage, cauliflower, radish, turnip, canola, horseradish, mustard	Characterized by pungent sulfur-containing compounds or "mustard oils" that are formed enzymatically from thioglucosides
Labiatae	Mint family—basil, marjoram, oregano, peppermint, rosemary, sage, savory, thyme	Herb plants, most of which are native to Mediterranean region; hair-like oil glands on leaves and stems contain "essential oils"
Umbelliferae	Parsnip family—celery, carrot, parsley, caraway, dill, fennel, coriander, anise, cumin	Herb and spice plants; seed-fruit are rich in "essential oils"
Solanaceae	Nightshade family—potato, tomato, eggplant, pepper	Characterized by toxic steroidal alkaloids in some nonedible species
Liliaceae	Lily family—alliums (onion, shallot, chive, garlic, leek), asparagus	Alliums characterized by lachrymatory, flavorful sulfur compounds that are formed enzymatically from *S*-alkylcysteine sulfoxides
Compositae	Daisy family—lettuce, endive, dandelion, chicory	Latex contains triterpenoid alcohols that act as soporific herbs
Rutaceae	Citrus family—lemon, orange, grapefruit, lime, citron, kumquat, mandarin	Flavonoid glycosides and terpenes contribute to characteristic flavor

caused by external sources such as molds, insects, and rodents. Perishable horticultural crops, on the other hand, generally are metabolically active plant parts, have high-moisture contents (70–95%), are large, have higher rates of respiration and heat production, are soft in texture, and have a shelf life of days to months. Losses for these commodities are usually due to internal factors such as rotting, physical damage, and senescence.

There are many ways that plant food crops can be handled and stored after they are harvested. Some are canned, frozen, pickled, dried, or milled to preserve their nutritional quality. These techniques may preserve the nutritive value and taste of the commodity, but the commodity is no longer living. However, there is a large demand for fresh horticultural commodities and their unique quality characteristics. To maintain these qualities, it is important to keep the product alive and realize the product is still respiring, and may undergo a variety of physiological, morphological, compositional, and color changes.

This chapter briefly covers many of the important principles of postharvest physiology, composition, and handling needed to successfully store and deliver plant foods to destination markets.

TABLE 17.3
Grouping of Edible Plants by the Organs That Make Up the Edible Portion

Plant Part	Examples
Entire plant	Beet, radish
Shoot	Green onion
Root	
Primary	Carrot, turnip
Secondary	Sweetpotato, cassava
Stem	Asparagus, kohlrabi
Tuber	Potato, yam
Leaf	
Mainly leaf blade	Leaf lettuce, spinach
Mainly petiole	Celery, rhubarb
Buds	Cabbage, head lettuce
Floral parts	Artichoke, broccoli, cauliflower
Bulb	Onion, garlic
Fruits	
Non fleshy, immature	Fresh peas, green beans, okra, sweetcorn
Non fleshy, mature	Seeds and nuts
Fleshy, immature	Cucumbers, summer (soft-rind) squash
Fleshy, mature	Apples, pears, peaches, berries, grapes, citrus, tropical fruits, melons, tomatoes, winter (hard-rind) squash

17.2 POSTHARVEST PHYSIOLOGY OF PLANT TISSUES

17.2.1 Morphology, Structure, Growth, and Development

A great diversity of plant parts and their structures are represented by harvested fruits, vegetables, seeds, and grains (Table 17.3). Knowledge of the structure of edible plant tissues is important to the food chemist for several reasons. It is important to recognize that an excised mass of tissue is not necessarily homogenous with respect to cell type, cellular organization, and distribution of chemicals. In addition, the nature and extent of chemical changes that occur in the tissues postharvest are partly, if not wholly, dependent on cellular organization. Each tissue is structurally adapted to carry out a particular function. Much of the metabolic activity of the plant is carried out in relatively unspecialized tissue called parenchyma, which generally makes up the bulk of edible plant tissues. The outer layer of the plant is called the epidermis and is structurally adapted to provide protection against biological or physical stress. Waxes on the surface of the epidermis tend to accumulate during development eventually creating a more or less impervious layer as in ripe fruits. Specialized tissues, called collenchyma and sclerenchyma, provide structural support. Water, minerals, and solute molecules are transported through vascular tissues: the xylem and phloem.

17.2.1.1 Plant Organs

Considering edible plant tissues according to the organ or organs that make up the edible portion of the plant (Table 17.4) is very useful in relation to postharvest considerations since, in most cases, those within a given group have similar postharvest behavior and requirements if harvested at a similar stage of development.

TABLE 17.4
Relationship Between General Structure of Horticultural Commodities and Their Postharvest Behavior

Group	General Postharvest Characteristics
Rapidly growing vegetative and immature fruit structures	Highly perishable Usually high respiration rate Rapid chemical changes Weight loss is a major cause of deterioration Continued growth can be a problem
Mature fruits	Vary in perishability from very high (strawberry) to low (apple) Undergo many physiological and compositional changes associated with ripening Decay can be an important deterioration factor
Fleshy storage organs and propagules	Low perishability Low respiration rate Growth can accelerate deterioration
Mature grains, seeds, and nuts	Very low perishability Very low respiration rate Moisture content is important to storage-life Germination can be a factor

17.2.1.1.1 Leaves and Petioles

A leaf is typically a flat and expanded organ, primarily involved with photosynthesis, but deviations in morphology and function may occur. The leaf is composed of epidermal tissue that is typically coated by a thin, waxy cuticle and contains specialized openings for gas exchange called stomata. Beneath the epidermal layer lies a series of elongated, closely packed palisade cells rich in chloroplasts, then irregular size parenchyma cells with large amounts of intercellular space. The vascular system consists of net-like veins in dicots and parallel veins in monocots. Petioles support the leaves on the plant, and conduct photosynthates from the leaf to the rest of the plant, and water and nutrients from the plant to the leaves. Leaves are metabolically active organs with usually very little stored energy, which makes them extremely perishable once they are removed from the plant. Lettuce, mustard greens, tea, chard, spinach, watercress, parsley, and cabbage are some examples of leaf crops.

17.2.1.1.2 Flowers

Flowers are plant reproductive organs, which in their immature stage are consumed as vegetables (e.g., broccoli and cauliflower). A flower contains the female portion or pistil and the male portion or stamen, surrounded by the calyx, which is made up of petals and leaf-like sepals. Immature floral tissues lose water easily because they have very little wax on their surface, and are among the most highly metabolically active parts of a plant, which makes them extremely perishable.

17.2.1.1.3 Fruits

Botanically, a fruit is a ripened ovary or the ovary and adjoining parts; that is, it is the seed-bearing organ. The flesh of the fleshy fruits may be developed from the floral receptacle, from carpellary tissue, or from extrafloral structures, such as bracts. Whatever its origin, it is generally largely composed of parenchymatous tissue with around 20% intercellular space. The anatomical features of some fruit types are illustrated in Figure 17.1. Cereals are a type of fruit called a caryopsis in which the thin fruit shell is strongly bound to the seed coat (Figure 17.2). The primary edible portion of edible seeds and nuts is the endosperm, which functions to store energy for the developing seed.

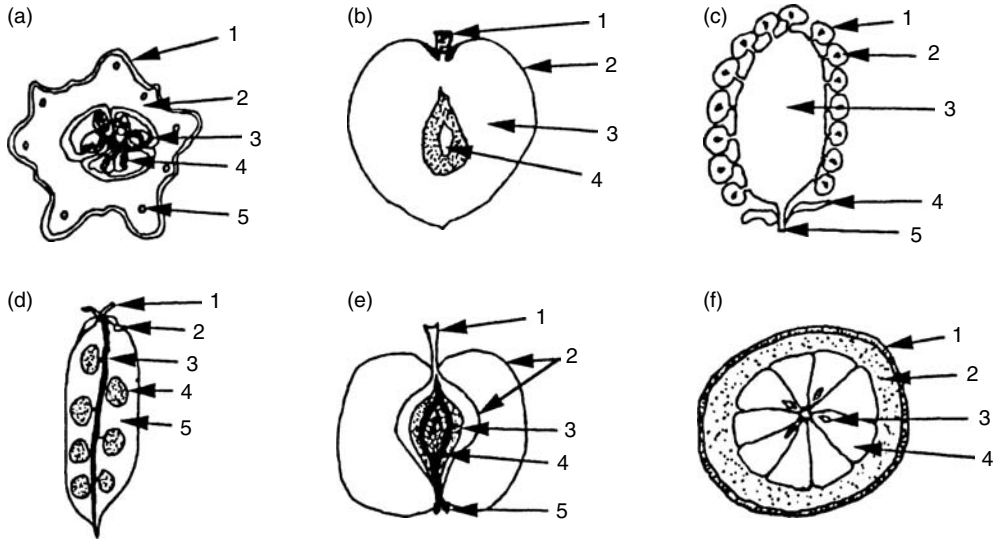


FIGURE 17.1 Diagrammatic illustrations of anatomical structures of different types of fruit. (a) Pepo (cucumber, squash, and pumpkin) in cross section: (1) rind (receptacular), (2) flesh (ovary wall), (3) placenta, (4) seed, and (5) vascular bundle. (b) Drupe (cherry, peach, and plum) in longitudinal section: (1) pedicel, (2) skin (ovary wall), (3) flesh (ovary wall), pit (stony ovary wall), and (5) seed. (c) Aggregate (raspberry, strawberry, and blackberry) in longitudinal section: (1) fleshy ovary wall, (2) seed (stony ovary wall plus seed), (3) fleshy receptacle, (4) sepal, and (5) pedicel. (d) Legume (pea, soybean, and lima bean) in longitudinal section: (1) pedicel, (2) sepal, (3) vascular bundles, (4) seed, and (5) pod (ovary wall). (e) Pome (apple and pear) in longitudinal section: (1) pedicel, (2) skin and flesh (receptacle), (3) leathery carpel (ovary wall), (4) seed, and (5) calyx (sepals and stamens). (f) Hespiridium (citrus) in cross section: (1) collenchymatous exocarp (flavedo), (2) parenchymatous mesocarp (albedo), (3) seed, and (4) endocarp of juice sacs formed by breakdown of groups of parenchyma-like cells.

The endosperm of legume seeds is absorbed by the embryo, which repackages the nutrients in the cotyledons to form the primary edible portion. The perishability of fruits varies widely; for fleshy fruits perishability is largely a function of maturity at harvest, with ripe fruits most perishable, while nonfleshy fruits may be highly perishable if they are developing seeds (e.g., peas and sweetcorn) or relatively less perishable if dry (i.e., cereals and nuts).

17.2.1.1.4 Stems

A stem consists of four distinct regions (from outside to inside): the epidermis, the cortex, the vascular system, and the pith, which forms the central core of cells (Figure 17.3a). The secondary tissues exhibit different forms as illustrated in Figure 17.3b and 17.3c. Most examples of edible stems are not above-ground support organs, but rather modified underground structures (corms, tubers, and rhizomes) such as potato and ginger, or bulbs (short stems bearing a series of fleshy leaves above and around the stem) such as onion and garlic. These modified stems serve a storage or perennating function in which energy stored as carbohydrates is held available for sprouting of the plant following a period of rest or dormancy. Their metabolic rate is low and they can be stored for extended periods (i.e., several months). An example of an above-ground edible stem is asparagus, which, in contrast to the storage organs above, is harvested as a rapidly growing shoot and is extremely metabolically active and consequently extremely perishable.

17.2.1.1.5 Roots

The basic anatomical structure of root tissue is illustrated in Figure 17.4. Fleshy roots are formed by secondary growth of the cambia (Figure 17.4b and 17.4c). The secondary vascular tissues of roots

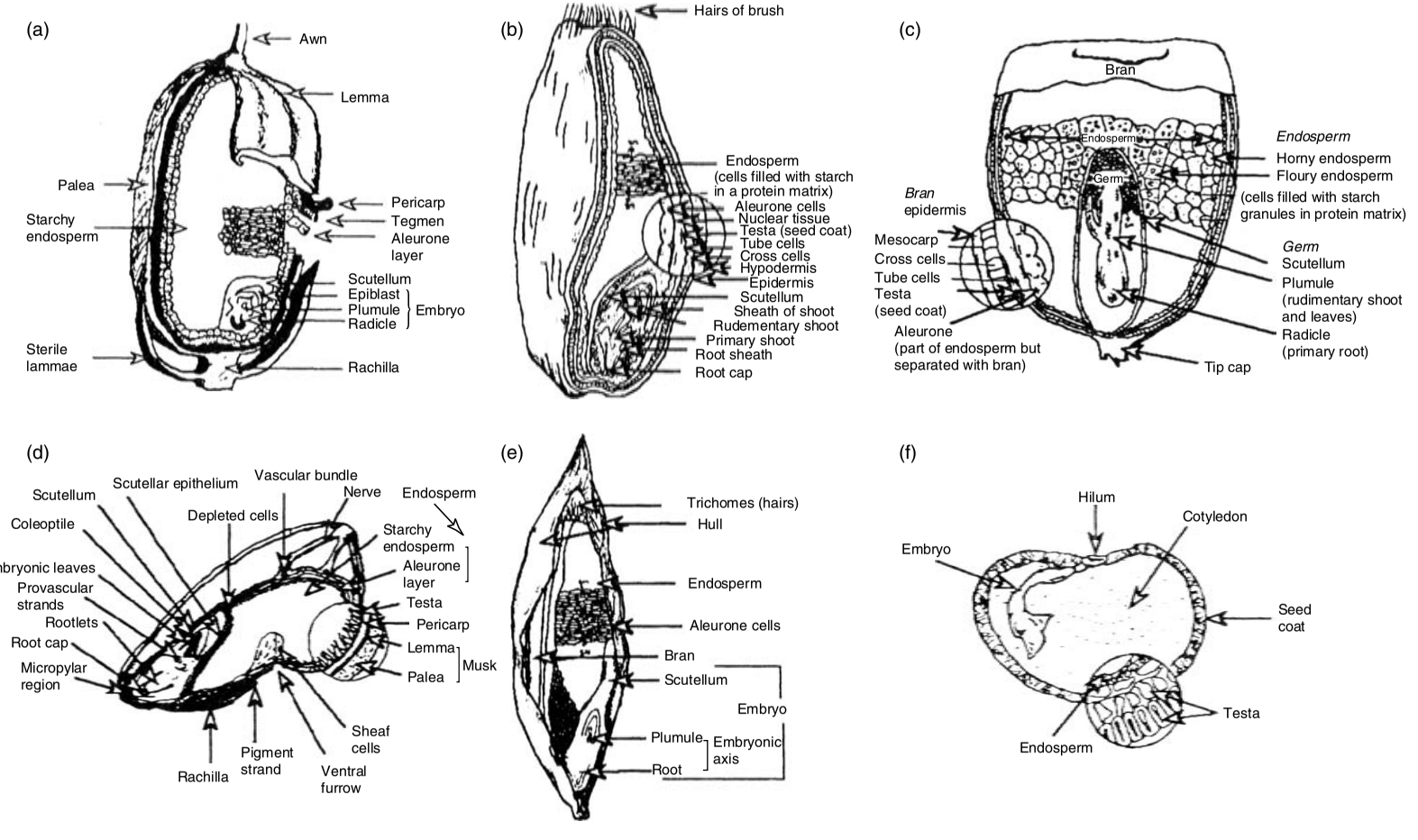


FIGURE 17.2 Diagrammatic illustrations of cereal grains called caryopsis fruit: (a) rice, (b) wheat, (c) corn, (d) barley, and (e) oat. An example of a true seed crop that does not contain a thin fruit shell is soybean (f). See also Figure 17.1d: 4.

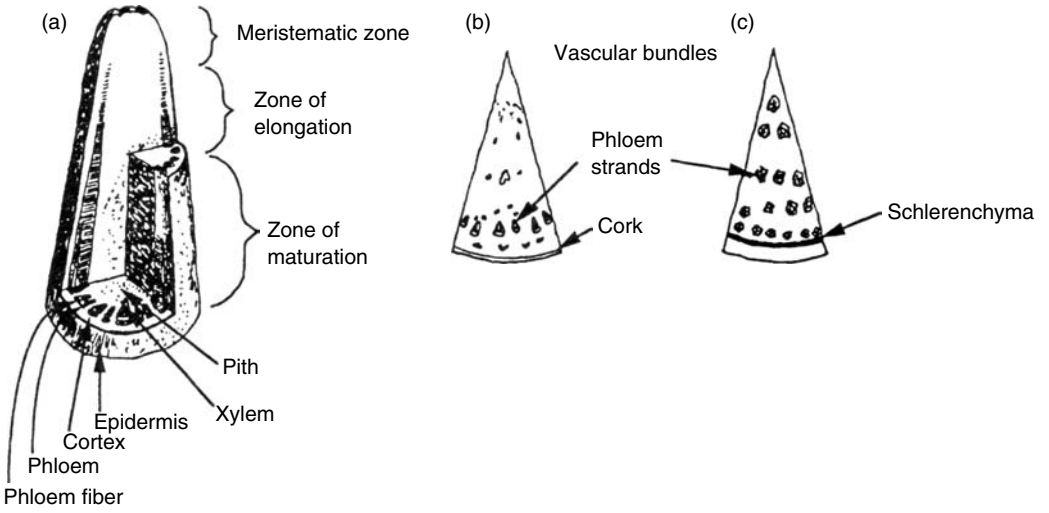


FIGURE 17.3 (a) Diagrammatic illustration of anatomical structure of stem tissue. (b) In dicotyledonous stem structures, such as potato, the vascular bundles are arranged in a single ring, as seen in transverse section. (c) Monocotyledons, represented by asparagus, show a characteristic scattering of vascular bundles throughout the parenchymatous ground tissue.

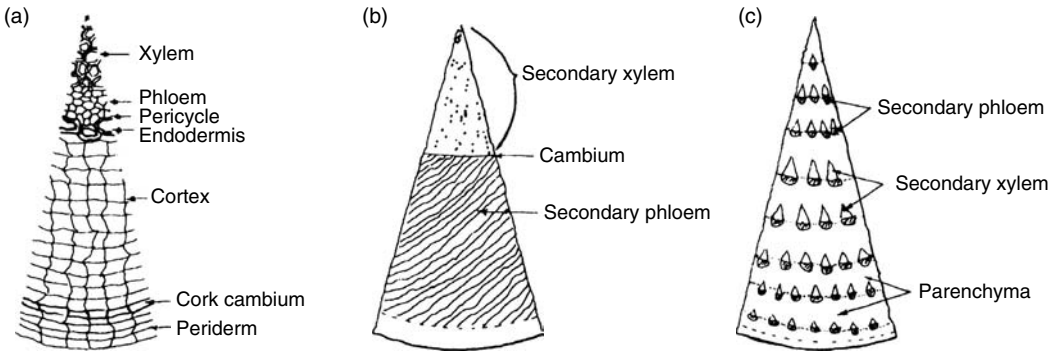


FIGURE 17.4 (a) Diagrammatic illustration of anatomical structure of root tissue in cross section. Fleshy roots are formed by secondary growth of meristematic tissue called cambia. (b) A typical example is carrot, in which the cambia forms phloem on the outside and xylem on the inside. The conducting elements are scattered throughout a mass of parenchyma tissue. (c) Beet root shows a different type of secondary growth, in which a series of concentric cambia are formed.

consist of small groups of conducting elements scattered throughout a dense matrix of parenchyma tissue with typically <20% intercellular air space. Multiple cambia are formed in certain tissues, such as in beet root (Figure 17.4c) and sweetpotato. Some crops that appear to be root tissue are mostly modified hypocotyl (e.g., radish) or stem (e.g., potato). Edible roots are storage organs that accumulate starch and sugars in parenchyma cells. Their perishability depends on if they are harvested before or after they finish accumulating storage reserves and are ready to enter the resting stage.

17.2.1.2 Cellular Structure

Plant cells are characterized by a rigid cell wall surrounding the cell and the presence of a large central vacuole (Figure 17.5). The cell wall acts as a cytoskeleton and its structure as well as postharvest

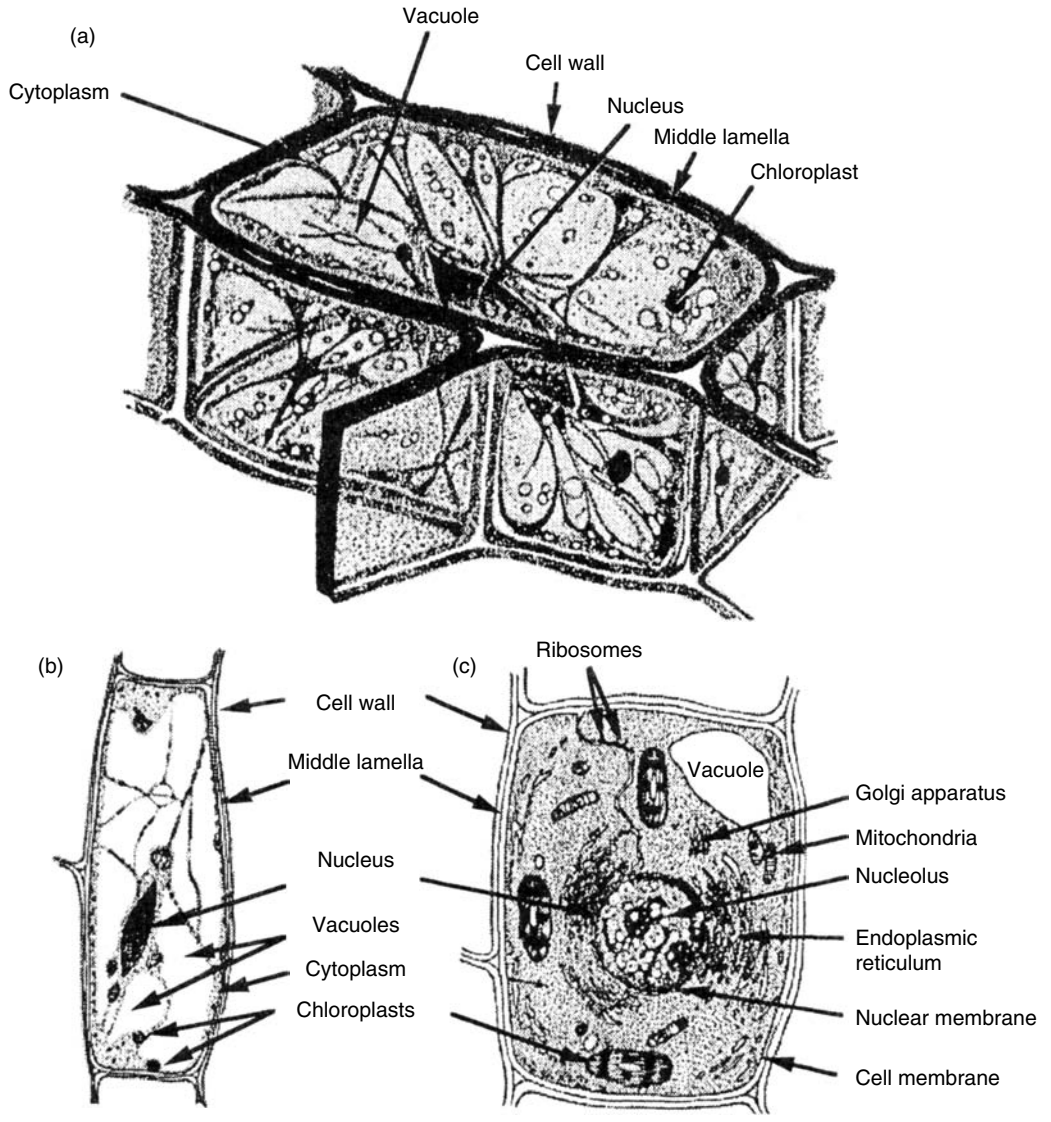


FIGURE 17.5 Diagrammatic representation of a mature plant cell. (a) Light microscope, (b) light microscope section, and (c) electron microscope image. (From Janick, J. R., W. Schery, F. W. Woods, and V. M. Ruttan (1969) Food and human needs, in *Plant Science* (J. Janick, R. W. Schery, F. W. Woods, and V. M. Ruttan, eds.), W. H. Freeman, San Francisco, CA, pp. 37–52.)

alterations to the cell wall play a large role in determining the textural properties of edible plant tissue. The cell wall is a source of dietary fiber. The vacuole of plant cells acts as a storage site for compounds such as sugars, organic acids, pigments, and phenolics that are important for the taste and appearance of fruits and vegetables. The vacuole also plays a role in regulating the turgor pressure of plant cells, which also contributes to textural properties. The cytoplasm of plant cells lies between the cell wall and vacuole and is bound by two membranes—the plasmalemma subtending the cell wall and the tonoplast surrounding the vacuole. Within the cytoplasm are found organelles including a distinct group in plants called plastids. Plastids are double-membrane bound and include chloroplasts, which contain the green pigment chlorophyll and the rest of the photosynthetic machinery, chromoplasts containing carotenoid pigments, and leucoplasts, one type of which (amyloplasts) is the storage site for starch in plant cells. Seeds may also contain protein bodies—single-membrane bound

organelles that store protein to be used by the germinating plant, but also nutritionally important in the human diet.

17.2.1.3 Growth and Development of Plant Parts

The series of physiological processes from the initiation of growth to death of a plant or plant part is termed "development" (Figure 17.6). The irreversible increase in physical attributes of a developing plant or plant part is, in turn, called "growth." In terms of the postharvest period for edible plant

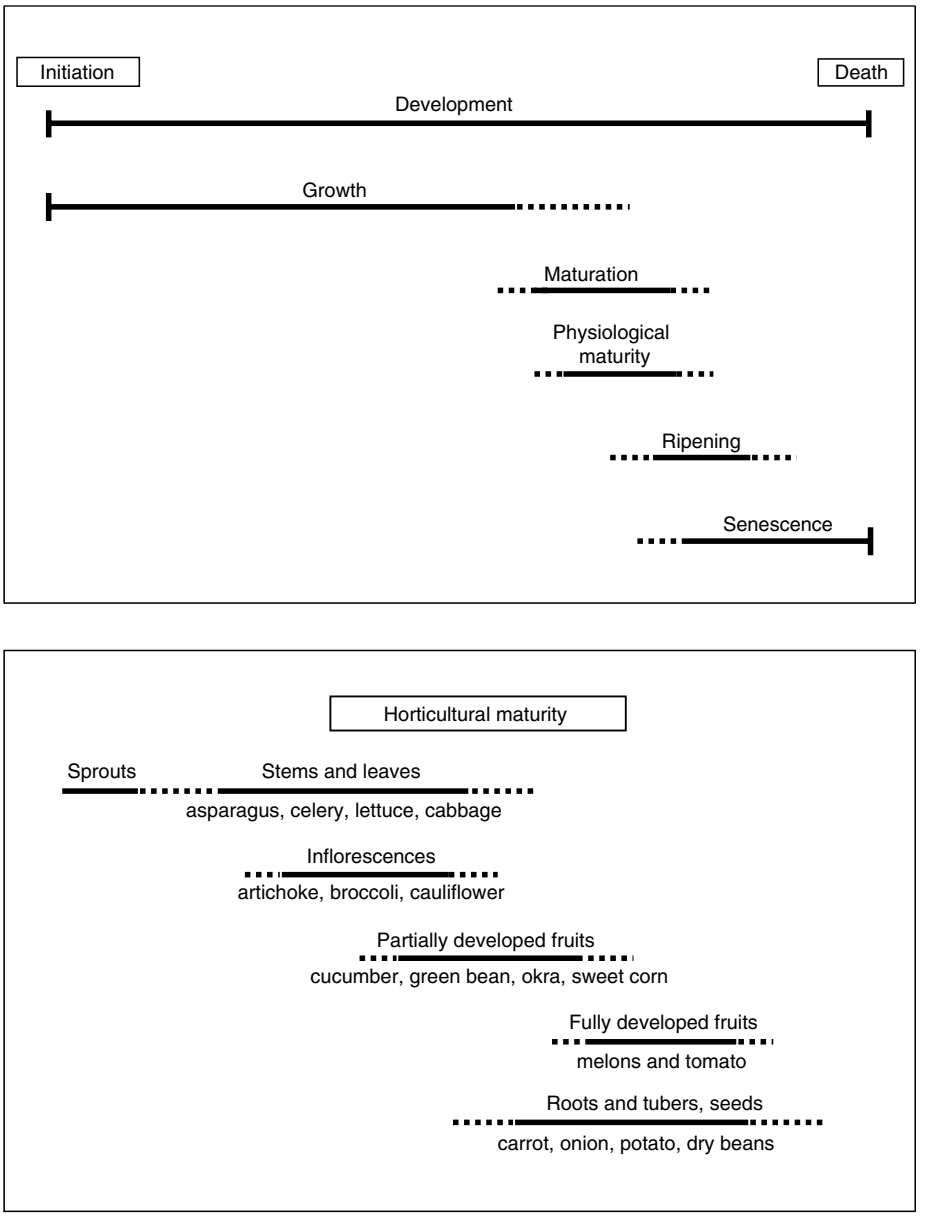


FIGURE 17.6 Horticultural maturity in relation to developmental stages of the plant. (Adapted from Watada, A. E., R. C. Herner, A. A. Kader, R. J. Romani, and G. L. Staby (1984) Terminology for the description of developmental stages of horticultural crops. *HortScience* 19:20–21.)

organs, the most important developmental process is that of "senescence." Other developmental processes may be cut short by the removal of an organ from the plant, but the process of senescence continues. Senescence consists of a series of genetically controlled changes that lead to the eventual death of cells, tissues, organs, and organisms. Fruit ripening is a special form of senescence familiar to most people. Much of the handling procedures and technology utilized in the postharvest handling of edible plant tissues has as its purpose the slowing of senescence processes.

Changes that can occur after harvest include growth—sprouting, rooting, elongation, and seed germination; toughening (due to increased lignification); increased fiber content; softening (due to changes in cell wall and loss of turgor); wound periderm formation (in response to wounding); and increased thickness of cuticle and wax deposits with fruit ripening.

The specific form taken by senescence processes in various plant organs is strongly influenced by the stage of development at which they are harvested. The developmental stage of edible plant organs at the time of harvest is referred to as their "maturity stage." An edible horticultural plant organ can be considered "horticulturally mature" (Figure 17.6) when it will fulfill the expectations of consumers for its intended purpose, which can occur at any stage of organ development depending on the crop. Thus, a bean sprout is horticulturally mature only a few days after the seed germinates while a potato tuber is not mature until it has completed its growth and entered a stage of dormancy. Horticultural maturity is distinguished from "physiological maturity"; the latter is related to the stage of development when the plant organ is able to continue its normal development whether attached or detached from the plant and usually corresponds to attainment of full size. Examples of physiologically mature plant organs are a fully expanded leaf that will subsequently senesce or a fully grown fruit that continues to ripen whether on or off the tree. The process of maturation can be thought of as the part of development that leads to physiological or horticultural maturity.

17.2.1.4 Diversity in Horticultural Maturity in Relation to Physiological Development

Horticultural maturity is a relative term because it can refer to any stage of development. For example, sprouts or seedlings are horticulturally mature very early in development; whereas, in vegetative tissues, inflorescences, fruits, and underground storage organs, horticultural maturity occurs somewhat later; and in seeds and nuts it occurs late in development (Figure 17.6). The stages depicted in Figure 17.6 for a specific type of morphological tissue also need to be considered on a relative scheme. For example, potato tubers are harvested after the plants have bloomed; whereas, carrots and onions are harvested before the plants have bloomed. Fruits have been separated into two groups, because some are harvested when they are partially developed and physiologically immature, while others are harvested when fully developed and physiologically mature, or even fully ripe. Stage of development is important in determining the time of harvest, the quality when harvested, and the behavior after harvest.

17.2.2 Respiration

Living organisms require complex metabolic processes to grow and reproduce. These chemical reactions both create (anabolism) important chemical molecules (e.g., sugars, proteins, fats, etc.) and break them down (catabolism) to release energy, primarily in the form of adenosine triphosphate (ATP). Even fresh horticultural products that are detached from the main plant continue metabolizing compounds in response to environmental conditions and genetic developmental programs until death through consumption or natural senescence processes. For example, after harvest, detached plant parts may continue respiration and ripening, or may heal minor wounds or produce defensive compounds to fight against fungal attack. The energy needed to carry out these processes comes primarily from aerobic respiration (with oxygen) whereby cells completely oxidize molecules (e.g., carbohydrates, organic acids, proteins, fats, etc.) to produce energy, carbon dioxide (CO_2), water,

and heat. In aerobic respiration, oxygen (O_2) serves as the final electron acceptor. During anaerobic respiration (without O_2), complete oxidation does not occur and electrons are instead passed on to other molecules within the cell. Respiration supplies the energy for all the other metabolic reactions so its rate directly reflects the overall metabolic rate of the commodity. Using a hexose sugar as a substrate, the overall equation for aerobic respiration can be expressed as follows:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 686 \text{ kcal}$$

When carbohydrates are used for aerobic respiration, about 1 mole of CO_2 is produced for each mole of O_2 consumed. The respiratory quotient (RQ) describes this relationship and is defined as

$$RQ = \frac{[CO_2] \text{ Produced}}{[O_2] \text{ Consumed}}$$

However, this ratio can change depending on what materials are being utilized for respiration. For example, when highly reduced lipids are being utilized for respiration, the $RQ < 1$, whereas $RQ > 1$ when organic acids are being utilized. Table 17.5 lists the RQs of several detached plant parts. The substrate utilized for respiration, and thus the RQ, is characteristic of the plant tissue and the type of respiratory substrate that is available.

Of the 686 kcal of energy liberated, ~41% is used to make ATP, ~57% is immediately lost as heat, and ~2% is lost during the conversion of the hexose into a simpler molecule. However, the energy used to make ATP will also ultimately be released as heat as the energy is passed to other molecules to perform work in the cell. Thus, an important byproduct of respiration is heat, which must be removed to prevent warming of the commodity [26].

When plant tissues are detached from their source of nutrients, water, and other resources, they can no longer replenish the substrates (carbohydrates, organic acids, etc.) used for respiration. Though the amount of plant dry weight lost to respiration is relatively small over the short-term, in long-term storage it can be significant (i.e., 1% per month).

TABLE 17.5
RQs for Some Detached Plant Parts

System	Respiratory Quotient[a]
Leaves rich in carbohydrate	1.00
Germinating starch seeds	1.00
Wheat seedlings in 5–20% O_2	0.95
Wheat seedlings in 3% O_2	3.34
Germinating linseeds	0.64
Germinating buckwheat seeds	0.48
Mature linseeds	1.22
Apples	
Preclimacteric apples	1.00
Climacteric apples	1.50
Postclimacteric apples	1.30
Apples treated with HCN	2.00
Apples treated with $CHCl_3$ vapor	0.25
Romaine lettuce	
Intact	1.05
After cutting	0.63

[a] Ratio of respiratory CO_2 production to O_2 consumption.

Commodities age both in regards to time (hours, days, months, etc.) and physiologically (how fast metabolic processes are taking place). The relative storage life of a commodity is tied more to its physiological age than to its temporal age. Thus, by reducing the metabolism (respiration) of a commodity, its quality is maintained longer, resulting in an extended storage life. The following are important factors to consider for maximizing the quality retention of living plant products after harvest.

17.2.2.1 Factors Affecting Respiration

Respiration rates vary depending on the type of commodity, stage of development, and ripening pattern. Different commodities inherently have different rates of respiration. For example, broccoli respires about 10 times faster than tomatoes at the same temperature. Because a commodity's rate of respiration is directly related to its storage life, high-respiring commodities such as asparagus, broccoli, and sweetcorn have correspondingly shorter storage lives compared with lower-respiring commodities such as watermelon, onion, and potato. Rapidly developing plant parts (e.g., asparagus, broccoli florets, etc.) exhibit high rates of respiration, which generally slow as development progresses. Conversely, dormant or slow-growing plant parts have very low rates of respiration. Because heat is a byproduct of respiration, high-respiring tissues produce more heat than low-respiring commodities, which must be removed to prevent commodity warming.

Following harvest, the respiration rate of fresh horticultural commodities either gradually declines over time, or first declines, but then experiences a sharp respiratory increase (called the "climacteric") before finally declining as the tissue senesces (Figures 17.7 and 17.8). A large increase in ethylene production precedes or is concurrent with the climacteric rise. Ethylene is a gaseous plant hormone that acts to promote and synchronize the senescence processes. Concomitant with the climacteric, climacteric fruits have a distinct ripening phase involving the conversion of starch to simple sugars, tissue softening, declining acidity, color change, and so forth. Table 17.6 lists various fruits and vegetables as having either a climacteric or nonclimacteric respiratory pattern. Most nonclimacteric

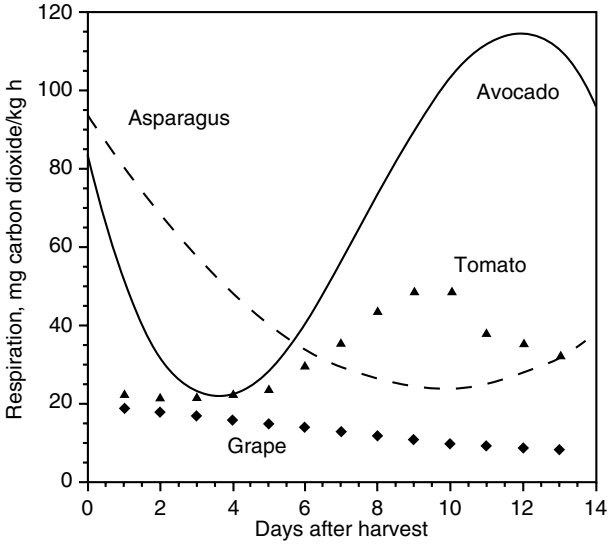


FIGURE 17.7 Respiratory patterns after harvest of a stem (asparagus), climacteric fruit (tomato and avocado), and nonclimacteric fruit (grape). The spontaneous respiratory burst in climacteric fruit is normally triggered by low concentrations of endogenous ethylene. Exogenous ethylene and wounding can stimulate respiratory bursts (not true climacteric) in many detached plant organs.

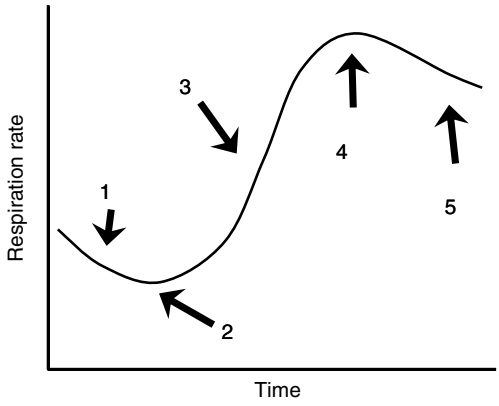


FIGURE 17.8 Phases of the respiratory climacteric: (1) preclimacteric, (2) preclimacteric minimum, (3) climacteric rise, (4) climacteric peak, and (5) postclimacteric phase.

TABLE 17.6
Classification of Edible Fruits and Vegetables According to Respiratory Patterns

Climacteric		Nonclimacteric	
Apple	Mangosteen	Blackberry	Olive
Apricot	Muskmelon	Cacao	Orange
Avocado	Nectarine	Cherry	Pepper (capsicum)
Banana	Papaya	Cranberry	Pineapple
Blueberry	Passion fruit	Cucumber	Pomegranate
Breadfruit	Peach	Date	Prickly pear
Broccoli	Pear	Eggplant	Pumpkin
Carambola	Persimmon	Grape	Raspberry
Cherimoya	Plum	Grapefruit	Squash
Durian	Quince	Jujube	Strawberry
Feijoa	Rambutan	Lemon	Tamarillo
Fig	Sapodilla	Lime	Tangerine
Guava	Sapote	Longan	Other leafy, succulent, and root vegetables
Jackfruit	Soursop	Loquat	
Kiwifruit	Sweetsop	Lychee	
Mango	Tomato	Okra	

commodities produce very small amounts of ethylene but are generally sensitive to ethylene exposure. Such exposure can cause physiological disorders and rapid deterioration.

17.2.2.2 Slowing Respiration and Increasing Storage Life

17.2.2.2.1 Temperature Management

Of the external factors, temperature has the greatest effect on respiration rate and proper temperature management is the most effective way to reduce respiration and prolong storage life of perishable horticultural commodities (Figure 17.9). For every 10°C increase in temperature, respiration generally increases two- to three-fold with a corresponding decrease in storage life. For example, sweet peppers respire about three times faster and have only about one-third of the storage life when held

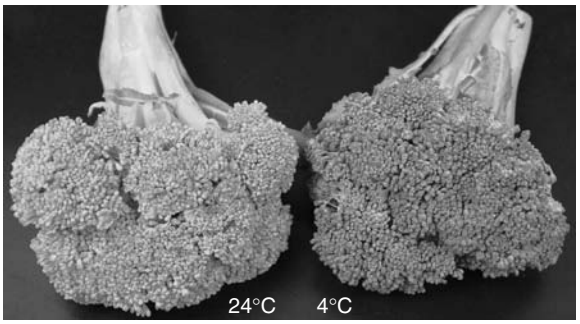


FIGURE 17.9 Effect of temperature on the quality of broccoli after just 48 h of storage near room temperature (24°C) or in the refrigerator (4°C). The lighter appearance of the broccoli stored at 24°C is due to accelerated chlorophyll breakdown.

at 20°C compared to holding at 10°C. The temperature coefficient over 10°C intervals (or Q_{10}) describes how the respiration of a commodity changes as the temperature is raised or lowered. Q_{10} values are usually highest between 0°C and 10°C ($Q_{10} = 2.5–4.0$), and decrease to 1 (no increase in respiration) as temperatures near the tissue's thermal death limit. When dealing with temperature intervals greater or less than 10°C, the following equation can be used to calculate Q_{10}:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2 - T_1)}$$

where T_1 and T_2 = temperatures (°C), R_2 = respiration rate at T_2, and R_1 = respiration rate at T_1.

If the temperature difference is 10°C, then

$$Q_{10} = \frac{R_2}{R_1}$$

Exposure to temperatures that cause tissue freezing or that are above the tissue's thermal death limit (between 50°C and 55°C) will usually kill the tissue. In addition, some commodities such as tomatoes, cucumbers, peppers, and so forth, are chilling sensitive and are injured by exposure to low but nonfreezing temperatures. Though respiration may be either elevated or depressed during exposure to chilling temperatures, chilled tissues of sensitive crops typically exhibit a burst of respiration upon warming. To maximize quality and storage life, commodities should be stored at the lowest temperature that does not result in chilling injury (CI) or freezing injury.

17.2.2.2.2 Gas Concentrations

Oxygen and CO_2 are key gases involved in respiration (and other metabolic processes within commodities). In general, reducing O_2 levels and/or increasing CO_2 levels (called modified or controlled atmospheres (CAs)—discussed later in this chapter) reduces the rate of respiration. Because plant tissues consume O_2 and evolve CO_2, simply sealing a commodity in an airtight container will begin to lower the O_2 and raise the CO_2 levels within the container. Even protective packaging (e.g., wraps) and commercially applied waxes can generate modified atmospheres (MAs) around and within commodities that may or may not be intentional. As O_2 levels continue to decrease and CO_2 levels increase, aerobic respiration slows to a point where it is no longer sufficient to meet the energy demands of the cell. At this point, anaerobic respiration begins to operate, supplementing total cellular energy production. As the tissue becomes more anaerobic, CO_2 production increases greatly because anaerobic respiration is much less efficient, producing only a fifth or less of the energy per CO_2 molecule released. The specific O_2 and CO_2 concentrations resulting in anaerobic respiration or tissue damage depends on the commodity, temperature, and so forth.

Other gases, such as ethylene or carbon monoxide, can also influence commodity respiration rates. For example, exposure to ethylene (or its analogs) hastens ripening, increases the respiration rate, and shortens the storage life of fresh horticultural commodities.

17.2.2.2.3 Physical or Physiological Stress or Injury

Any type of physical abuse (e.g., from drops, punctures, and abrasions) or stress (e.g., dehydration, pathogen attack, etc.) will also cause respiration to rise quickly and storage life to be shortened.

17.2.3 Gene Expression and Protein Synthesis

Expression of genetic information is an event of general and fundamental importance in postharvest plant food crops. Physiological processes such as sprouting of tubers and bulbs, germination of seeds, fruit ripening, and plant senescence are developmental cascades resulting from transcription of DNA and translation of mRNA to proteins (enzymes). Increased levels of specific enzymes have been noted to occur in numerous plant organs following harvest. For example, aldolase, carboxylase, chlorophyllase, phosphorylase, peroxidase, phenolase, transaminase, invertase, phosphatase, *O*-methyltransferase, catalase, and indoleacetic acid oxidase are among the enzymes known to emerge in ripening fruit. Many of these enzymes arise as a result of *de novo* synthesis and have a relatively short half-life *in situ*. It is generally observed that plant senescence is accompanied by increased RNA synthesis and an increased rate of transcription. Indeed, there appears to be an absolute requirement of continued protein synthesis for ripening fruit. For example, cycloheximide, an inhibitor of protein synthesis, also inhibits degreening, softening, ethylene biosynthesis, and ripening of ethylene-treated banana fruit [10]. Increased protein synthesis and nucleic acid synthesis are most pronounced at the early stages of fruit ripening. The propagation of slow-to-ripen or ripening-impaired mutant varieties of fruit is consistent with findings that fruit ripening is under genetic control [63]. Moreover, genes for key enzymes that promote plant senescence have been cloned and their expression blocked by antisense technology [48]. These findings show that postharvest deterioration of plant tissues can be controlled by manipulation of gene expression.

17.2.3.1 Recombinant DNA Technology and Genetic Engineering of Plant Crops

The discovery of DNA restriction (cut) and ligase (join) enzymes in the 1970s opened the way for developing methods to alter the genome of plants. Isolated genes can be modified by site-directed mutagenesis to achieve a target protein with different properties. In addition, foreign DNA can be introduced into an organism (transformation), such as a plant, and the new plant is called a transgenic. While there has been intensive interest in genetically engineering plants for crop improvement [7,19], applications to date have primarily focused on herbicide, insect, and virus tolerance during plant production in the field.

Various genetic engineering strategies have been used to improve plant crops. For example, in a May 1994 milestone decision, the U.S. Food and Drug Administration approved Calgene's Flavr Savr tomato, the first genetically engineered food [39]. Flavr Savr tomatoes were genetically engineered to contain the antisense gene for polygalacturonase (PG), which is one of several enzymes synthesized during the ripening of tomato fruit that catalyzes breakdown of pectin and contributes to tissue softening. During ripening of Flavr Savr, the normal expression of the tomato PG gene is blocked (>95%) and the softening process is delayed by several days. Many other applications of genetic engineering to improve the sensory, nutritive, and processing characteristics of fruits and vegetables are under development or review for approval.

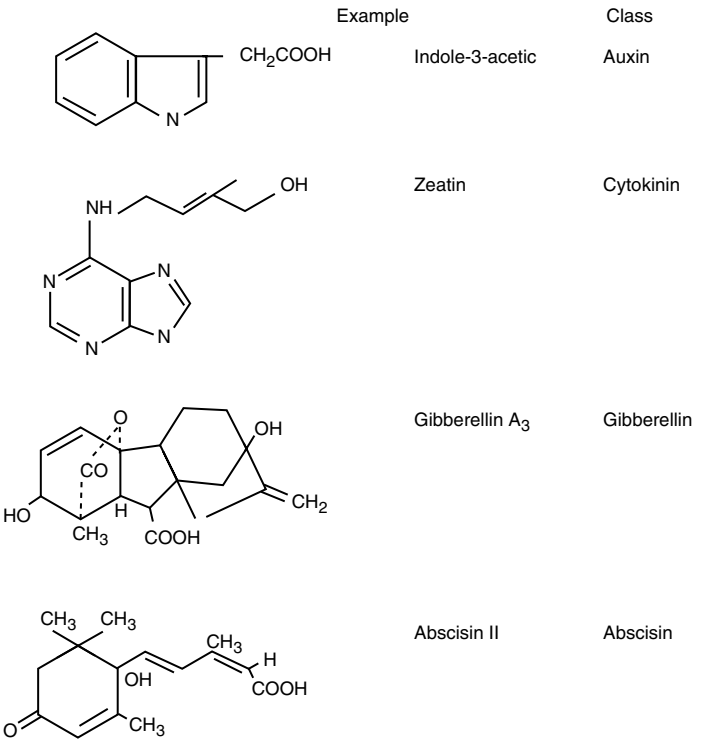


FIGURE 17.10 Some plant hormones and their structures. Other hormone and hormone-like substances, such as jasmonic acid, have recently been identified in plant tissues.

17.2.4 Plant Hormones

Senescence of plant tissues and organs as well as other developmental processes are controlled and regulated by interactions among the various plant hormones (i.e., auxins, gibberellins, cytokinins, ethylene, and abscisic acid). Plant hormones are active at very low concentrations as important regulators of physiological events in detached plant organs.

Of the five major categories of plant hormones (Figures 17.10 and 17.11), ethylene has received the most attention by postharvest physiologists. The ability of this agent to "trigger" ripening of climacteric fruits and generally promote plant senescence has led to the descriptive name "the ripening hormone." Ethylene can also induce sprouting in potato tubers and is also described as a "wound hormone" because stresses such as physical or chemical damage, fungal elicitation, and γ-irradiation stimulate ethylene production. Gibberellin, which increases amylase production during malting of grains and increased size in table grapes, is the only other plant hormone that is used commercially with plant foods.

17.2.4.1 Roles of Plant Hormones in Plant Development

Auxins, gibberellins, and cytokinins are considered as retardants of senescence, while ethylene and abscisic acid are regarded as senescence promoters. The former are associated with growth and development by regulating cell division, enlargement, and maturation, while the latter antagonize the activities of the other hormones. Application of auxins, cytokinins, or gibberellins can simulate the action of juvenility factors in desensitizing plants to exogenous or endogenous ethylene. There is also evidence that the abscisins, which play a role in leaf or organ abscission from the parent plant, also act to promote other senescence phenomena such as fruit ripening. As with other physiological

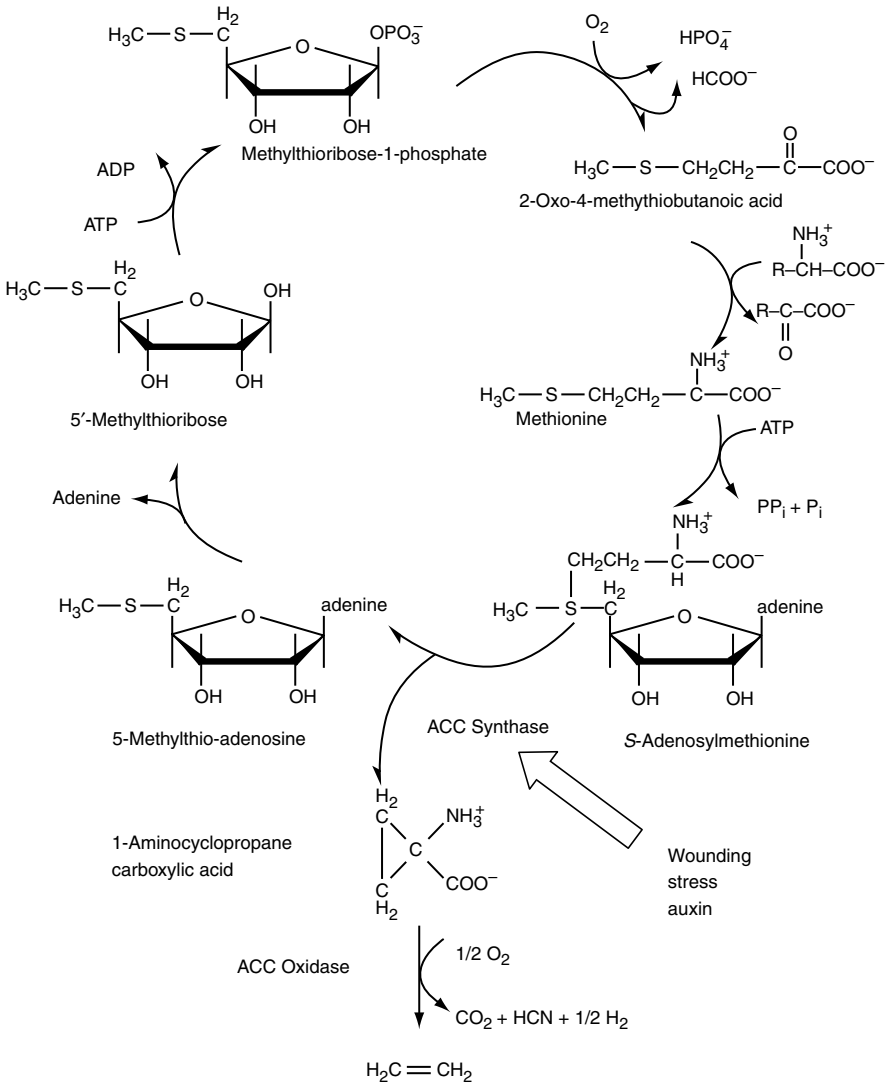


FIGURE 17.11 The Yang Cycle of ethylene biosynthesis. The sulfur-containing amino acid methionine is recycled and is the carbon source for the key intermediate 1-aminocyclopropoane carboxylic acid (ACC).

events, such as seed germination, it appears that plant hormones act in concert to control physiological events in postharvest commodities.

The cell, tissue, and organ concentrations of hormones in plants are under continuous flux throughout development. Plant hormone concentrations in plants are regulated by transport from one part of the plant to another, by synthesis and degradation or metabolism, by deactivation via conjugation and, in the case of the gaseous hormone ethylene, by diffusion from the tissue. Available data on the natural levels of the various hormones in fruits point out the diversity among species, the large changes that occur during development and senescence, and lack of close correlations between the levels of extractable hormones and the stage of development. Because tissue sensitivity to the hormones also changes during development, it is possible for the activity of a hormone to change with no corresponding change in hormone concentration. Coupled with the fact that the role of each class of hormones may be direct or indirect through effects on other

hormones, elucidation of the mechanisms of hormone control in plant development is exceedingly difficult.

17.2.4.2 Ethylene

Ethylene is a simple, gaseous hydrocarbon and is produced essentially in all parts of the plant during its development. Both these features differentiate ethylene from other "classical" plant hormones, which are synthesized in specific tissues or organs and transported elsewhere in the plant to exert their effects. Although metabolism of ethylene to form ethylene glycol and ethylene oxide occurs, its concentration in the plant is regulated almost exclusively by its rates of synthesis and diffusion.

Ethylene is a ubiquitous compound, being naturally produced by plants, soil microbes, plant pathogenic microbes, natural gas, and burning vegetation; man-made sources of ethylene include combustion of coal and oil, refuse burning, internal combustion engines, cigarette smoke, fluorescent lights, and rubber materials exposed to heat or ultraviolet (UV) radiation. Ethylene is flammable in air at concentrations between 3.1% and 32% by volume.

17.2.4.2.1 Ethylene Biosynthesis

Ethylene biogenesis in plant tissues involves conversion of *S*-adenosylmethionine (SAM) to 1-aminocyclopropane 1-carboxylic acid (ACC) by the enzyme ACC synthase and the subsequent conversion of ACC to ethylene by ACC oxidase. Ethylene synthesis requires oxygen, which explains to a large degree the beneficial effect of reduced oxygen MA and CA technology in inhibiting fruit ripening. Methionine is regenerated by the methionine cycle [75] (Figure 17.11). The cyanide that is produced in conjunction with ethylene is detoxified in a reaction catalyzed by cyanoalanine synthase [36]:

$$\text{L-Cysteine} + \text{cyanide} \xrightarrow{\text{Cyanoalanine synthase}} \beta\text{-Cyanoalanine} + H_2O$$

Several inhibitors of ethylene biosynthesis are known, particularly, aminoethoxyvinylglycine and aminooxyacetate, which inhibit ACC formation from SAM, and salicylic acid and cobalt, which inhibit conversion of ACC to ethylene.

Climacteric fruit show a large increase in ethylene production during ripening and all plant tissues respond to wounding and other stress by increasing the production of ethylene. Ethylene formation associated with climacteric fruit ripening and following wounding results from stimulation of existing mRNA for ACC synthase. This autocatalytic feature of ethylene synthesis necessitates removal of even trace quantities of this gas from the storage environment to avoid early fruit ripening. It has long been recognized that ethylene acts (along with several analogs) to stimulate respiration in both climacteric and nonclimacteric crops, but the stimulation of respiration in nonclimacteric species is dependent on the continuous presence of exogenous ethylene while that of climacteric species continues without exogenous ethylene once autocatalytic ethylene synthesis is initiated. Negative feedback inhibition of ethylene biosynthesis is also known, occurring in nonclimacteric tissues and immature climacteric organs that are not yet physiologically competent to initiate ripening.

17.2.4.2.2 Ethylene Action

Ethylene reversibly binds to ethylene-specific receptors (five are known: ETR1&2, ERS1&2, and EIN4) on the plasma membrane, which are transmembrane proteins (histidine kinases with a copper cofactor) [47]. Binding of ethylene to these receptors is reversible, thus requiring excess ethylene molecules to saturate the response. The ethylene receptors are negative regulators of ethylene action. That is, they act to prevent constitutive ethylene responses in the absence of ethylene and ethylene binding "de-represses" the response pathways. Binding by ethylene to the receptor inactivates a response regulator protein (CTR1, a protein kinase). CTR1 is normally a negative regulator of ethylene responses via negative regulation of a membrane transporter protein (EIN2). Inactivation

of CTR1 apparently initiates a protein phosphorylation cascade, activating EIN2. The activated EIN2 initiates a transcription cascade via two families of transcription factors (EIN3/EIL → ERF1), which bind to specific promoters in the nucleus to turn on gene expression leading to ethylene physiological responses.

17.2.4.2.3 Avoiding Ethylene Effects

The most practical procedure for avoiding ethylene effects when handling fruits and vegetables is to exclude ethylene from the postharvest environment, such as by using electric forklifts (no exhaust), removing ripening and decaying plant material, and avoiding mixing ethylene-producing commodities with those that are sensitive to ethylene. Alternatively, ethylene may be removed from the environment by ventilation or by ethylene "scrubbers." The most common chemical ethylene scrubber is potassium permanganate (alkaline $KMnO_4$ on inert pellets), which oxidizes ethylene, while activated and brominated charcoal absorbs and oxidizes ethylene. Ozone or UV light used to produce ozone ($O_2 + UV \rightarrow O_3$), is also used to oxidize ethylene:

$$C_2H_4 + [O] \rightarrow\rightarrow CO_2 + H_2O$$

In this system, UV at 185 nm produces ozone, which oxidizes ethylene, while UV at 254 nm destroys the remaining ozone, which is harmful to plant tissues at very low concentrations. Systems that catalytically oxidize ethylene using platinum or oxide catalysts plus heat (200–300°C) and low temperature (~100°C) catalysis with TiO_2 + UV are also available. Finally, low pressure storage systems (hypobaric storage) facilitate ethylene removal from fruit and vegetable tissues by increasing the diffusion gradient (see Section 17.2.5).

Compounds that compete with ethylene for binding at the ethylene receptors and thus inhibit ethylene responses by fruits and vegetables have long been of great theoretical and practical interest among plant physiologists. Silver ion is a potent inhibitor of ethylene action, irreversibly binding to and blocking the ethylene receptor. Silver thiosulfate complex is commonly used to prevent ethylene damage on cut flowers and ornamental plants, but cannot be used on food crops. Other inhibitors of ethylene action include carbon dioxide, norbornadiene, and 1-methylcyclopropene (1-MCP), which are gaseous compounds that, like silver, competitively inhibit ethylene action [59]. The inhibitory effects of each of these compounds wears off within a few days to two weeks depending on the compound, the treatment dose, storage temperature, and so forth. Besides dissociation of the inhibitors from the receptor, this loss of inhibition has been associated with synthesis of new receptor proteins. Thus, turnover of receptors is probably an important component of the changes in sensitivity to hormones observed during plant development.

17.2.4.2.4 Ethylene and Fruit Ripening

The amounts of ethylene produced by different fruits vary greatly (Table 17.7). Climacteric fruits produce large amounts of ethylene coincident with ripening, but nonclimacteric fruits produce little ethylene during their development and ripening. Production of ethylene by ripening climacteric fruits is closely related to, but may occur before or after, the climacteric rise in respiration. Fruit ripening is not always accompanied by a clearly defined increase in ethylene to threshold levels. This is probably related to variable changes in tissue sensitivity to ethylene among different fruit species since the efficacy of exogenous ethylene to stimulate such events as ripening can depend on the receptiveness of the tissue. For example, the concentration of ethylene required to initiate fruit ripening declines as the fruit approaches full growth. Similarly, fruit attached to the plant may display less sensitivity to ethylene than detached fruit. Also, certain cultivars of pear are sensitized to ethylene only after the harvested fruit are stored at low, nonfreezing temperatures. These observations have been used to support the suggestion that "juvenility factors" desensitize fruit to ethylene and may otherwise control physiological events in immature fruits and vegetables. The development of transgenic plants that do not synthesize ethylene [45,48] unequivocally showed that ethylene is a trigger rather than a

TABLE 17.7
Classification of Horticultural Commodities According to Their Ethylene Production

Class	Range at 20°C (μL C_2H_4/kg-h)	Commodities
Very low	0.01–0.1	Cherry, citrus, grape, strawberry, pomegranate, leafy vegetables, root vegetables, and potatoes
Low	0.1–1.0	Blueberry, cucumber, okra, peppers, persimmon, pineapple, and raspberry
Moderate	1.0–10.0	Banana, fig, honeydew melon, mango, and tomato
High	10.0–100.0	Apple, apricot, avocado, cantaloupe, feijoa, kiwifruit, nectarine, papaya, peach, pear, and plum
Very high	>100.0	Cherimoya, passion fruit, sapote, and mammee apple

byproduct of climacteric fruit ripening. Ethylene is widely used commercially to ripen climacteric avocadoes, bananas, and tomatoes and to "degreen" nonclimacteric grapefruit and oranges. Ethylene treatment promotes faster and more uniform ripening in climacteric fruits and faster ripening can mean reduced time between harvest and consumption of fruits, which also means better quality and nutritive value for the consumer.

17.2.4.2.5 Ethylene and Other Physiological Processes in Postharvest Crops

Ethylene accelerates ripening and softening of fruits, and accelerates senescence and loss of green color in leafy vegetables and some immature fruits (e.g., cucumbers, squash). Ethylene causes chlorophyll degradation by stimulating the expression of the gene for chlorophyllase, although neither ethylene nor chlorophyllase are universally required for chlorophyll degradation to occur. A CA helps retain the green color of vegetables, presumably through competitive inhibition of ethylene action by the elevated concentration of carbon dioxide and inhibition by the reduced concentration of oxygen of metabolism in general and specifically ethylene synthesis. There is little evidence that ethylene directly affects pigments other than chlorophyll.

Ethylene also stimulates abscission of leaves in some heading vegetables (e.g., cauliflower, cabbage), formation of bitter-tasting isocoumarin in carrots, lignification of *Brassica* species and asparagus, production of phytoalexins in diseased crops, production of stress metabolites in sweetpotato and potato, onset of russet spotting in lettuce, and onset of hard core in sweetpotato. It also reduces the incidence of CI in muskmelons and other crops if applied prior to low temperature storage by advancing ripening or senescence, which reduces chilling sensitivity. In cabbage, ethylene accelerates losses of weight and sugar and promotes changes in organic acids.

17.2.5 Transpiration and Water Loss

Water (H_2O) has many unique properties that make it a vital ingredient for life on earth. The unequal sharing of electrons between oxygen and hydrogen is responsible for its strong polar nature and gives rise to hydrogen bonding between water molecules and many other unique characteristics. For example, water's polarity gives it the ability to dissolve more substances than any other common liquid, thus serving as an ideal medium for most biochemical reactions within the cell. The high tensile strength and surface tension of water allow it to be pulled (by evaporation from leaves) to plant parts even at the top of tall trees. In addition, water's low compressibility gives rise to turgor pressure within cells imparting the firm, crisp texture of well-hydrated fresh commodities that consumers desire.

The water content of plant products has profound effects on their postharvest quality and storability. On one end of the spectrum are cereal and seed crops, which must be kept under relatively dry conditions to maintain low (e.g., <14%) water content. Storage in humid environments promotes higher mold, sprouting, and respiration. On the other end of the spectrum, fresh horticultural commodities are composed mostly of water (generally 90–95%). This section will focus primarily on transpiration and minimizing water loss from fresh horticultural commodities. Within these plant tissues, even small amounts of water loss (e.g., <1%) can cause physiological changes yielding negative impacts on quality and storability. Water loss leads to reductions in appearance quality (e.g., wilting, shriveling, accelerated development of injuries, etc.), textural quality (e.g., loss of crispness, juiciness, etc.), and even nutritional quality (e.g., accelerated loss of vitamins A and C). Most fresh fruits and vegetables become unsalable after losing 3–10% of their weight. Furthermore, water represents most of the salable weight of fresh fruits and vegetables. Therefore, water loss equates with lost sales for those commodities sold on a weight basis.

Following harvest, fresh commodities are separated from the plant body and cannot replace lost water. Thus, reducing water loss after harvest is critical for maintaining quality and the salable weight of the product. Water escapes from fresh horticultural commodities through evaporation into the surrounding air. Even undamaged commodities will lose some water during postharvest handling and storage.

The driving force for water loss is the concentration gradient between water vapor inside the commodity's intercellular spaces and water vapor in the surrounding atmosphere. The final rate of water loss will also be influenced by various barriers to water movement (e.g., cuticular waxes, trichomes, stomates, etc.) that might be present. To understand the process, we must first define a few important terms.

Vapor pressure (VP) is the partial pressure of water vapor in the air (in kPa, mm Hg, etc.). Water moves from areas of high water VP, to areas of low water VP.

Saturated vapor pressure (SVP) is the VP of saturated (100% RH) air at a given temperature. Air within the intercellular spaces of a commodity is considered to be at SVP.

Vapor pressure difference (VPD) is the difference between the SVP and the VP in the surrounding air ($VPD = SVP_{tissue} - VP_{air}$). The greater the VPD, the greater the driving force for water loss from a commodity.

For example, using a psychrometric chart or table to look up VP values, the air within a room at 20°C with 45% RH will have a VP = 1.1 kPa. The SVP of air inside a commodity at the same temperature is 2.3 kPa. Thus, VPD = 2.3 − 1.1 = 1.2 kPa. Raising the room RH to 90% will increase the room VP to 2.1 kPa and reduce the VPD, and hence the driving force for water loss, to VPD = 2.3 − 2.1 = 0.2 kPa. Therefore, the lower the RH of air surrounding a commodity, the greater the VPD and the faster water is lost. In most instances, maintaining RH between 85% and 95% is recommended. When higher RH (i.e., 95–100%) is required, cartons must be constructed of water-resistant materials to prevent carton deterioration. The use of 100% RH is only recommended when commodities and containers can tolerate free water (i.e., broccoli) from washing, hydrocooling, or condensate.

Lowering the storage temperature also greatly reduces VPD and water loss. If the air at 45% RH in the previous example was at 0°C, instead of 20°C, then VP = 0.3 kPa, SVP = 0.6 kPa, and VPD = 0.3 kPa, instead of 1.2 kPa. Therefore, water loss can be minimized by quickly cooling the product, keeping the commodity and surrounding air as cool as possible without causing product injury, and by minimizing fluctuations in commodity and air temperatures during handling and shipping operations.

The rate of water loss (J; reported as %/day, g/h, etc.) can be calculated from the VPD and a proportionality constant (K) using the equation: $J = K \times VPD$. The proportionality constant takes into account all factors other than VPD—including the physiological state of the commodity

and anatomical barriers to water diffusion (e.g., cuticular waxes, trichomes, stomates, etc.). After empirically measuring J for a commodity under known VPD conditions, K can be calculated and then used to predict water loss of the same commodity under different conditions (e.g., during cooling, at lower or higher temperatures, etc.).

Commodities with a large surface area (e.g., leaves) are much more prone to water loss than compact, round commodities (e.g., potatoes). Natural barriers to water loss, such as a waxy cuticle or abscission layer, also help prevent water loss. For example, the lack of open lenticels in tomato peel makes them less susceptible to water loss than cucumbers, which is why cucumbers are waxed or shrink wrapped. Also, cuts or abrasions that break these natural barriers will increase water loss. Thus, careful handling to reduce injury is the first step in reducing commodity water loss.

Even when room RH is relatively low, a layer of saturated air (known as a boundary layer) forms around the commodity, which inhibits water vapor from diffusing into the rest of the room. Pubescent hair on the surface of some commodities helps maintain this boundary layer and reduces water loss. Packaging such as plastic wraps and liners inside packed cartons serve the same purpose. Conversely, rapid air movement around a commodity breaks up the boundary layer and maintains a relatively steep VPD gradient for water loss. Thus, once a commodity is cool, minimizing air movement around the commodity allows the boundary layer to re-form around the commodity to slow water loss. Some practical methods that can be used to reduce water loss in fruits and vegetables are listed in Table 17.8.

17.2.6 Plant Stress Responses

Throughout its life cycle, the intact plant must respond to various physical and chemical challenges from the environment. A plant's reaction to stress or environmental change is normally in the direction of protection, for example, prevention of water loss, adaptation to temperature extremes, or development of physical and chemical barriers to pathogens. Diverse stresses tend to activate similar responses, which typically include the production of stress proteins and antioxidant metabolites [11]. When the challenge is such that the tissue cannot successfully adapt, the response may be one of hypersensitivity manifested as gene-directed death of particular groups of cells. Antioxidant compounds in plants such as ascorbic acid, carotenoids, flavonols, phenolic acids, and tocopherols exist to protect the plant from oxidative and other stresses, but are increasingly recognized for their

TABLE 17.8
Methods to Reduce Water Loss

Handle fresh fruits and vegetables carefully
Cure/dry certain root, bulb, and tuber vegetables to allow natural tissues that restrict water loss to develop at the site of wounds
Use waxes and other surface coatings to further restrict water loss
Quickly cool commodities and maintain temperatures at the lowest safe temperature (i.e., non-chilling or freezing temperatures)
Minimize fluctuations in commodity and air temperatures
Reduce fan speeds in storage rooms after the commodity has cooled. Use of wraps, plastic liners, and other packaging also slows water loss
Add moisture to the storage room air through the use of humidifiers or by wetting the room floor and maintain RH at the highest recommended level without causing commodity or container deterioration
Design cooling systems so that the evaporator coil temperature is within 1°C of the air temperature
Use crushed ice in shipping containers and mist systems in retail displays of commodities that tolerate direct contact with water (e.g., leafy vegetables, snap beans, peas, sweetcorn, etc.)
When the CO_2 tolerance of a commodity is known, it is often possible to leave the fresh-air exchange opening on trailers and containers closed or partially closed, which allows higher RH to be maintained

important dietary role in protecting humans against chronic diseases such as atherosclerosis, coronary heart disease, and certain cancers [43,67].

It is important to recognize that specific responses of different species of plants may differ markedly in response to physical, chemical, and biological challenges. This is important because it means that no single condition or treatment is ideal for extending the storage lives of all fruits and vegetables. Before and after harvest, the quality of perishable commodities can be reduced by a variety of improper environmental or cultural conditions. Fruits and vegetables are also subject to direct physical forces during handling that result in cuts, scrapes, bruises, and so forth. Disorders without an obvious causal fungal, bacterial, viral, or insect agent are termed "physiological disorders." Physiological disorders can be broadly classified into two groups—those caused by adverse temperature conditions and those apparently resulting from physiological malfunction within the normal temperature range for the product.

17.2.6.1 Physical Damage

Wounding of plant organs generally results in a temporary, localized burst of respiration, formation of ethylene, a rapid turnover of certain cellular constituents, and sometimes accumulation of specific secondary metabolites or products that appear to have a protective function. In fleshy organs, wounded cells commence synthesis of messenger RNA and proteins. Although the impact of wound physiology on plant food commodities is not fully understood, sufficient examples exist to illustrate the general and practical significance of these events.

17.2.6.1.1 Protective Substances

The response of plant organs to stress may be accompanied by "wound healing," that is, formation of a physical barrier of protective substances. Formation of a waxy or suberized barrier, or of a lignified layer, is important in certain commodities since these barriers can effectively prevent invasion by saprophytic microorganisms and subsequent spoilage. Accordingly, following harvest of certain crops, such as potatoes, it is desirable to cure or store the crop under environmental conditions conducive to rapid wound healing. These conditions usually differ from those yielding maximum storage lives after wound healing is complete. Wound healing is of particular concern with mechanically harvested commodities, since this can result in extensive surface abrasion. In certain crops, such as asparagus, the cut injury associated with harvesting may be especially problematic since the resulting stimulation of lignin deposition is directly associated with the losses in tenderness and succulence.

17.2.6.1.2 Stress Metabolites

Wound injury can also lead to synthesis and accumulation of a diverse family of substances called "stress metabolites" [23]. In certain cases, accumulation of a stress metabolite occurs only when mechanical injury is accompanied by other conditions such as the presence of ethylene or exposure to chilling temperatures. Stress metabolites encompass a broad family of chemical compounds, including isoflavonoids, diterpenes, glycoalkaloids, and polyacetylenes (Figure 17.12), and they appear to serve a protective function for the plant by virtue of their antibiotic properties. However, they may also detract from food quality because of their bitter taste (e.g., coumarins in carrot root, furanoterpenes in sweet potato root, or glycoalkaloids in potato tuber) and because some have toxic properties. Certain stress metabolites are also precursors of enzymic browning reactions.

17.2.6.2 Temperature Extremes

17.2.6.2.1 Freezing Injury

Reduction of the ambient temperature below that of the freezing point of the tissue can result in freezing injury, a disorder with symptoms that include water-soaked areas in the tissue, and collapse

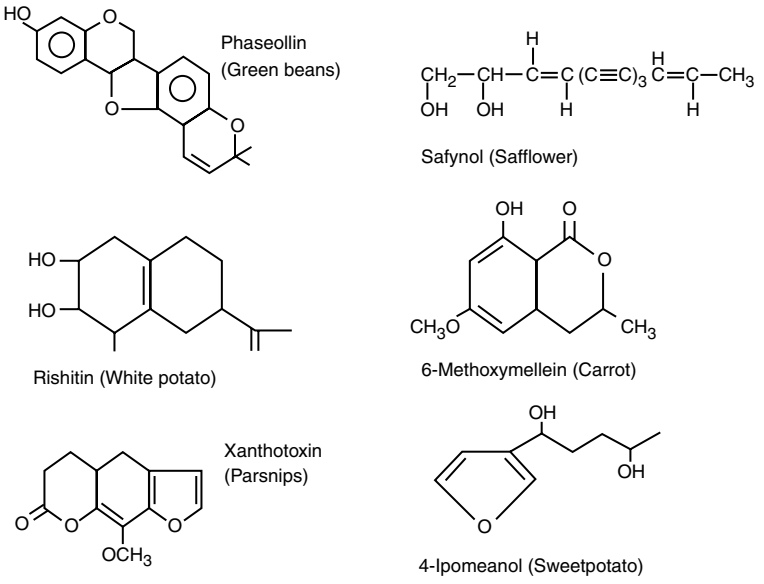


FIGURE 17.12 Examples of stress metabolites identified in edible plants. The compounds shown are representative of many other compounds that accumulate in stressed plant tissues and are called "phytoalexins" (to ward off) by plant pathologists because of their antibiotic activity. In sufficient quantities some of these compounds are toxic to mammals.

TABLE 17.9
Freezing Points for Some Common Fruits and Vegetables

Commodity	Freezing Point Range (°C)
Apple	−2.2 to −1.7
Asparagus	−1.4 to −1.1
Cherry	−4.3 to −3.8
Cucumber	−0.9 to −0.8
Grape	−5.3 to −2.9
Lettuce	−0.6 to −0.3
Onion	−1.3 to −0.9
Orange	−2.3 to −2.0
Potato	−1.8 to −1.7
Tomato	−1.0 to −0.7

and even disruption of the epidermis. Low temperatures may occur in the field or in situations where temperatures in the storage environment fall, whether through faulty refrigeration thermostats, or extreme low temperature during transportation.

The presence of dissolved solids in the cell sap reduces the freezing point of plant tissues below that of pure water. This freezing point depression, which is a function of the osmolality of the cell solution, is at most a few °C and often less than 1°C (Table 17.9). High-sugar wine grapes may not freeze until the temperature falls below −5.0°C, while leafy vegetables such as lettuce can freeze just below 0°C. A knowledge of the way in which freezing occurs in plant tissues is important in understanding the symptoms that may develop.

When the temperature of tissues falls below the freezing point, ice crystals are not immediately formed because there is a substantial capacity for supercooling in plant tissues; for some time, the cell solution remains liquid even though it is below its freezing point. In many situations, short periods of exposure to freezing conditions will not damage tissue because it supercools and rewarms without ice ever being formed. In certain situations, however, supercooling may be devastating because of the rapidity with which freezing occurs when the supercooled solution finally freezes, either by prolonged exposure to low temperature or when nucleated by vibration. Sudden freezing of supercooled tissues probably involves freezing of the cytoplasm and vacuolar sap, and is therefore always lethal. On thawing, such tissues become water-soaked and limp, and become targets for fungal infection.

If nucleation occurs before too much supercooling has taken place, extracellular ice crystals will form in the liquid in the cell wall and free space. In this more typical situation, the growth of the ice crystals in the cell wall liquid increases the VPD across the cell membrane, and the ice crystals continue to grow at the expense of the liquid in the cell, which may become plasmolyzed. Freezing of this type is not necessarily lethal, depending on the effects of the dehydration on the plasma membrane and on the risk of puncturing membranes when ice crystals grow into the space left inside the wall by the plasmolyzing cytoplasm. The natural freeze-drying occurring in this process reduces the freezing point of the cell sap by increasing its osmolality, thereby making the tissue more resistant to intercellular freezing. In tissues with abundant intercellular air spaces, extracellular ice crystals may form without disturbing the cells. On thawing, such tissues will initially appear water-soaked due to the increased liquid in the intercellular spaces, but can reabsorb the water, provided the plasma membrane is still intact.

17.2.6.2.2 Chilling Injury

Most plant species of subtropical or tropical origin, and even a few of temperate origin, show symptoms of a physiological disorder called chilling injury (CI) if they are exposed to temperatures below a critical threshold, but still well above their freezing point [70]. The extent of CI is dependent on both temperature and duration of exposure, but exposure of a susceptible commodity to a temperature below the chilling threshold for more than short periods causes injury and a decrease in both quality and shelf life. CI can occur in the field, during transportation, at the market, or in the home; and the harmful effect is additive and cumulative. Recovery is known to take place from short exposures to potentially harmful temperatures. In some cases, intermittent warming during storage can help prevent symptoms of CI, perhaps by allowing metabolism or detoxification of toxic compounds.

This disorder, which is characterized by increased susceptibility to fungal attack, collapse and necrosis of tissues, water soaking, and death, is particularly important in commodities of tropical and subtropical origin. Other symptoms of CI include flesh discoloration, as in low-temperature breakdown of apples and peaches; vascular discoloration, as in avocado and banana; epidermal pitting, as in citrus fruits, cucumbers, peppers, and tomatoes; and necrosis of seeds, as in peppers and tomatoes. The "threshold temperature" for CI is characteristic of a commodity, and is that temperature above which no injury is seen, regardless of the length of the storage period. Threshold temperatures are very commonly in the 10–15°C range, but can vary from quite low (e.g., 3°C for some apple varieties) to quite high (in some seasons, pineapples grown in Queensland, Australia have been injured at temperatures as high as 20°C).

There has been much interest over the years in the cause of CI. Whether or not there is a universal biochemical mechanism of CI is not known. Development of CI symptoms is, nonetheless, generally attributed to an imbalance in metabolic reactions resulting in underproduction of certain essentials and overproduction of metabolites that become toxic to the tissue. For example, accumulation of ethanol, acetaldehyde, and oxaloacetic acid are associated with CI of certain fruits. The observation by Raison et al. [54] of a sharp break in the rate of mitochondrial respiration plotted on an Arrhenius plot, suggested a sudden change in the activation energy of some key enzyme in respiratory metabolism at that point. The observation that the threshold temperature was close to the temperature at which

membranes of chilling-sensitive plants changed from a liquid to a crystalline state (the so-called phase transition) suggested that this phase change was perhaps a primary event in the chilling of sensitive plants, and might itself change the activation energy of lipid-associated respiratory enzymes. With more detailed research, this simple picture has been somewhat modified, but it still seems likely that changes in membrane fluidity, structure, and function are early events in CI.

Very soon after the chilling temperatures are sensed by the cell, there is a dramatic change in cytoplasmic architecture and cytoplasmic streaming stops. Prolonged exposure to reduced temperatures causes profound dislocation of streaming and of the cytoplasmic strands when the tissue is rewarmed. These events may also be of crucial importance in the symptoms of CI. The inability of the cell to transport substrates, metabolites, and control molecules could easily result in metabolic imbalance and accumulation of toxic respiratory intermediates.

Differences in the temperature sensitivity of important regulatory enzymes such as phosphofructokinase have been found between closely related species that differ in their sensitivity to CI. Because of the critical role that regulation of these enzymes plays in the regulation of metabolism overall, it may be that their malfunction could also be a cause of the symptoms of CI.

Plants must continuously prevent and/or quench the formation of reactive oxygen species (ROS), including superoxide anions (O_2^-), hydroxyl ions (•OH), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). ROS can damage critical cellular targets, such as DNA, lipids, and proteins. Peroxidation of membrane lipids, which is commonly observed in plants exposed to chilling stress, may also be involved in the development of CI. Chilling-sensitive plants may be unable to mobilize antioxidant defenses or enzyme repair systems when exposed to temperatures below their critical threshold.

Besides differences in chilling susceptibility among plant species, various factors can influence the chilling sensitivity of a given species or cultivar. Sensitivity to CI can be influenced by growing conditions and by stage of physiological development (advancement of maturity, ripeness, and senescence tends to cause fruits and vegetables to be less sensitive to CI). In some crops, short periods of exposure to low, nonchilling temperatures "acclimate" the tissues to chilling temperatures and reduce their sensitivity to CI. Briefly exposing fruits such as tomato and mango to temperatures around 38–55°C reduces their sensitivity to CI. Effective exposure times vary from a few days at the lower end of the range to few minutes at the upper end. High-temperature-induced resistance to CI may be related to redirection of protein synthesis toward heat-shock protein synthesis, which could protect the fruits from CI by reducing synthesis of other stress-induced proteins. In their role as molecular chaperones, heat-shock proteins may also help protect other proteins from cold-induced disruption of folding, unfolding, assembly, or disassembly.

Other techniques used to reduce CI include storage in CA, especially with elevated CO_2, and infiltration of fruit with Ca^{2+} under vacuum, both of which significantly reduce CI development. It seems likely that these techniques treat the symptoms of CI rather the primary cause, since both CA and Ca^{2+} tend to slow metabolic deterioration, which it stands to reason would include those involved in the development of CI-induced dysfunctions.

17.2.6.2.3 High Temperature Injury

Exposure of plant tissues to abnormally high temperatures can also produce characteristic injuries in specific commodities. For example, storage of many fruits above 30°C results in failure to ripen normally and exposure of apples to 55°C for a few minutes gives the fruit an appearance similar to that resulting from CI. High temperatures can inhibit key enzymes and thus disrupt normal metabolism (e.g., ripening). In many crops, exposure to direct sunlight on or off the plant can raise tissue temperatures to injurious levels. In some circumstances, as in "green-shoulder" of tomatoes, the tissues fail to ripen. High temperatures in ripening rooms may cause "sticking" of bananas, which means that the fruit fail to ripen properly. Higher tissue temperatures will cause actual death of cells, and result in collapsed and bleached areas on the commodity. Fruits, especially those that have been harvested, are particularly sensitive to sunburn because of their reduced capacity for evaporative cooling. Hot-water dips, sometimes used for fungal control, can also cause surface injury in fruits.

17.2.6.3 Ionizing Radiation

Low doses of ionizing radiation have been successfully applied experimentally and commercially to prolong the storage life of certain fresh fruits and vegetables. Inhibition of sprouting in potatoes, onions, and carrots, as well as destruction of insect pests in stored fruits, vegetables, and grains can be accomplished with 0.1–0.25 kGy doses. Control of bacterial pathogens can be accomplished with 0.7–1 kGy doses, while control of mold growth requires doses in excess of 1 kGy. Kilogray doses of ionizing radiation can temporarily or permanently prevent the onset of fruit ripening. Higher doses of radiation, which irreversibly prevent normal fruit ripening, interfere with the ability of the tissue to repair radiation damage. Low doses (<1 kGy) of irradiation cause a temporary burst in wound ethylene, but they also inhibit the subsequent stimulation of ethylene production and the activity of ACC oxidase.

Unfortunately, the quality of fruits and vegetables is usually adversely affected by doses of ionizing radiation in excess of 1–2 kGy, resulting in adverse textural and flavor changes. Moreover, low doses employed for sprout inhibition can interfere with wound healing, and this has created problems with commercial attempts to use this technique to prevent sprouting of potatoes.

The physiological changes induced by irradiation, especially at high doses, can bring about the death of cells. Irradiated commodities often show characteristic symptoms of damage. For example, apples exposed to 10 kGy exhibit a ring of necrotic tissues, not unlike core flush, adjacent to the core. Citrus and banana exhibit peel lesions at doses as low as 0.25 kGy. Many commodities also exhibit extensive pulp softening, and this has been related to activation of pectic enzymes. Despite these difficulties, there appears to be renewed interest in the use of ionizing radiation for preservation of fresh fruits and vegetables due to the impending ban on the use of methyl bromide (an ozone-depleting chemical) for quarantine-required insect fumigation and rising concerns about protecting the food supply from both inadvertent and intentional contamination with human pathogenic microbes. As with other techniques, the preceding limitations can probably be overcome when the influences of variables such as maturity, temperature, and gaseous environment are more fully controlled.

17.2.6.4 Nutritional Disorders

A number of disorders that affect fruits and vegetables before or after harvest have their origins in poor supply of nutrients to the developing plant organ. Calcium in plants is involved in a number of fundamental physiological processes that influence the structure of cell walls, membranes, and chromosomes and the activity of enzymes. In addition, some studies indicate that endogenous calcium may also serve an important regulatory function in fruit ripening. Little is known about how calcium exerts a beneficial effect when applied to detached plant tissues. Several commercially important physiological disorders are the result of poor calcium nutrition. These include bitter pit of apples and cork spot in pears, blackheart of cabbage, and blossom end rot of tomatoes and melons. In some fruit crops, the presence of cork-like areas in the flesh, usually at harvest, is the result of boron deficiency. Often, however, these disorders are not readily apparent at harvest, appearing only after some period of storage. Control of nutrient-related disorders can be achieved in a number of ways, by the selection of resistant varieties, application of the chemical as field sprays or postharvest (i.e., calcium dips and vacuum infiltration), and by harvesting at the proper maturity. CA storage reduces the incidence of bitter pit and other storage disorders of apples.

17.2.6.5 Disorders Resulting from Environmental Toxicants

A range of surface blemishes can be the result of exposure of commodities to a number of toxic chemicals used postharvest. Important ones are ammonia (used as a refrigerant), SO_2 (from coal-fired plants for generating electricity and also used as a fungistat in grapes), $CaCl_2$ (used for controlling bitter pit), and methyl bromide (used for insect fumigation). These disorders usually appear as dark spots on the surface of the commodity, where cells have died and collapsed. Exposure of fruits and

TABLE 17.10
Ranges for Percent Proximate Composition of Crops Represented in Major Economic Groups of Edible Plants

Group	Water	Carbohydrates	Lipids	Proteins
Fruits	80–90	5–20	0.1–0.5	0.5–3
Vegetables	80–90	2–20	0.1–0.3	5–7
Pulses, seeds	10–50	6–60	1–18	5–25
Nuts	3–50	10–40	2–70	3–25
Cereals	12–14	65–75	2–6	7–12

vegetables to various other chemicals can influence physiological processes in an undesirable way. For example, postharvest commodities show different susceptibilities to air pollutants and this can be of practical concern in urban areas. Automobile emissions, especially ethylene, can accelerate tissue necrosis at parts per million levels. The volatile components of photochemical smog (O_3, peroxyacyl nitrates, and NO_2) are known to create undesirable injury symptoms in a wide array of fruits and vegetables.

17.3 CHEMICAL COMPOSITION OF PLANT TISSUES

Detached plant parts, like all living organisms, contain a wide range of different chemical compounds and show considerable variation in composition. The proximate composition for the major economic groups of edible plants is summarized in Table 17.10. Apart from the obvious interspecies differences, an individual plant organ, largely composed of living tissues that are metabolically active, is more or less constantly changing in composition. The rate and extent of chemical change can depend on the growing conditions prior to harvest, the physiological role of the plant part, the genetic pool of the cell, and the postharvest environment.

The composition of plant foods is important because of their nutritional contribution to the human diet (Table 17.11; [20]). Knowledge of plant food composition and compositional changes is also important to understand how such things as harvest maturity, metabolic processes, and postharvest handling can affect their nutritional quality. Plant foods supply energy in the human diet mainly by supplying carbohydrates. Most fruit and vegetables are rich sources of vitamins, minerals, and dietary fiber. A few, like avocadoes and nuts are rich in oils, while grains, seeds, and nuts may be significant contributors of proteins in our diet.

Many factors influence the composition of edible plant tissues. Genetic factors influence the potential levels of various compounds in different cultivars, and rootstocks can influence the genetic potential of the scion. Preharvest environmental factors such as climate, temperature, light, and pollutants; cultural factors such as soil type, nutrient and water supply, thinning, and spacing; the maturity stage of the plant organ at harvest, fruit ripeness, or physiological age; and postharvest environmental factors, handling methods, and the duration between harvesting and consumption all influence the composition.

17.3.1 Major Components of Edible Plant Foods

17.3.1.1 Water

Given an unlimited supply of water, the moisture content of viable plant tissue assumes a characteristic maximum value associated with a state of complete turgor of the component cells. Water generally represents about 80–95% of the fresh weight of horticultural crops (i.e., fruits and

TABLE 17.11
Percent Nutritional Contribution of Edible Plant Foods Relative to the Total Food Supply

				Macronutrients					
Foods	**Food Energy**	**Carbohydrates**	**Dietary Fiber**	**Protein**	**Total Fat**	**Saturated Fat**	**Monounsaturated Fat**	**Polyunsaturated Fat**	**Cholesterol**
Legumes, nuts, and soy	3.1	2.1	14.3	6.3	3.8	2.1	4.0	5.6	0
Grain products	23.9	38.7	36.1	22.2	2.5	1.7	1.3	4.4	0
Fruits	3.3	6.2	11.8	1.3	0.5	0.1	0.5	0.5	0
Vegetables	4.8	8.4	26.5	5.4	0.5	0.2	0.1	1.0	0
Total	**35.1**								

				Vitamins					
	A	**Carotenoids**	**E**	**C**	**Thiamin**	**Riboflavin**	**Niacin**	**B_6**	**Folate**	**B_{12}**
Legumes, nuts, and soy	0.1	0.1	5.6	0	4.3	1.5	3.9	3.6	16.1	0
Grain products	9.0	0.7	4.5	5.6	59.7	40.2	45.7	19.8	37.5	0.1
Fruits	3.2	6.6	3.6	41.0	3.6	2.3	2.1	9.8	9.5	0
Vegetables	36.4	82.2	7.3	47.5	8.8	5.1	10.0	20.7	19.1	0
Total	**48.7**	**89.6**	**21.0**	**94.1**						

				Minerals					
	Ca	**P**	**Mg**	**Fe**	**Zn**	**Cu**	**K**	**Na**	**Se**
Legumes, nuts, and soy	4.4	6.1	13.4	7.6	5.5	19.7	9.3	0.3	7.1
Grain products	4.9	19.1	23.0	53.6	27.9	22.6	9.0	0.8	39.6
Fruits	2.6	1.9	6.4	2.5	1.2	6.5	11.2	1.8	0.5
Vegetables	6.5	7.5	14.0	9.8	6.3	18.6	26.4	27.7	2.5
Total	**18.4**								

Source: USDA. 2002. Nutrient Database for Standard Reference, Release 14. U.S. Dept. of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Nutrient Data Laboratory, Beltsville, MD (CD-ROM).

vegetables) and less than 20% of the weight of agronomic crops such as cereals, nuts, and pulses. The juiciness and succulence of fruits and vegetables is directly related to their high water content, and loss of water by transpiration causes undesirable changes in texture and mouthfeel. Apart from the influence of water loss on wilting and weight loss, one must recognize that moisture balance can indirectly evoke undesirable or desirable physiological changes in some crops. The interaction of water with other molecular constituents and its role in cellular metabolism are discussed in Chapter 2.

17.3.1.2 Carbohydrates

Aside from water, carbohydrates are the most abundant and widely distributed food component derived from plants. In general, approximately 75% of the dry matter of plants is carbohydrate, consisting primarily not only of simple sugars and polysaccharides, but also including pectic substances and lignin. In plants, carbohydrates are localized in the cell wall and intracellularly in plastids, vacuoles, or the cytoplasm. The amount of carbohydrate found in different plant foods varies widely, from as low as 2% of the fresh weight in some leafy vegetables, to 10–25% in some dessert fruits and more than 30% in starchy vegetables, and over 60% in some pulses and cereals.

The structural framework, taste and food value of edible plant foods are related to their carbohydrate content. Sucrose, glucose, and fructose are the main sugars in horticultural crops. Dessert fruits and certain vegetables are relatively high in sugars. Polysaccharides include starch, cellulose, hemicellulose, and pectin. Starch serves as a storage carbohydrate and is organized into small grains within the cell. The polysaccharides are the main structural components of cell walls and are important in texture and softening. The energy-yielding forms of carbohydrates that accumulate in plant tissues are primarily either starch (vegetables and cereals) or the soluble sugars (fruits).

Changes in carbohydrates of horticultural crops after harvest are among the most important from the standpoint of quality. For example, sugar loss due to respiration or conversion of sugars to starch results in loss of sweetness. Conversely, conversion of starch to sugars in ripening fruits results in desirable sweet flavor while starch to sugar conversion in potatoes at low temperatures results in undesirable darkening when the potatoes are thermally processed into chips, crisps, and French fries.

17.3.1.2.1 Cell Wall Constituents

The principal cell wall constituents are cellulose, hemicelluloses, pectins, and lignin. Cellulose is one of the most abundant substances in the biosphere, is largely insoluble, and is indigestible by human beings. The hemicelluloses are a heterogeneous group of polysaccharides that contain numerous kinds of hexose and pentose sugars and in some cases residues of uronic acids. These polymers are classified according to the types of sugar residues predominating and are individually referred to as xylans, arabinogalactans, glucomannans, and so forth. Pectin is generally regarded as α-1,4-linked galacturonic acid residues esterified to varying degrees with methanol. Pectins found in the primary wall have a higher degree of methylation than do pectins in the middle lamella between cells. Interaction of the carboxyl groups of pectin with calcium can form ionic cross-links between the polymer chains. Purified pectin invariably contains significant quantities of covalently linked non-uronide sugars, such as rhamnose, arabinose, and xylose, which result in the "hairy" region of the molecule. The albedo (white spongy layer) of citrus peel is an especially rich source of pectin, containing up to 50% of this constituent on a dry weight basis. Commercially, apple and citrus peel are major sources of pectin, with the latter being used primarily as a gelation agent. Lignin, always associated with cell wall carbohydrates, is a three-dimensional polymer composed of phenyl propane units, such as syringaldehyde and vanillin, and these are linked through aliphatic three-carbon side chains. Lignification of cell walls, notably those of xylem and sclerenchymatous tissues, confers considerable rigidity and toughness to the wall. Although generally ignored, cell walls also contain small amounts of structural proteins and enzymes. The best characterized structural protein is extensin, which occurs in the wall, but not the middle lamella [15]. Most of the enzymes that have been identified in the wall are peroxidases and hydrolases.

Cell wall constituents are the primary components of "dietary fiber." The biological availability of protein and other nutrients [65] can be reduced by these constituents, but there is considerable evidence for the beneficial role played by fiber in health and disease. The structure and chemistry of these substances are discussed in more detail in Chapter 3.

Despite rapid advances in analytical methodology and molecular biology, the precise molecular details of cell walls that are needed to explain softening during ripening and expansion during growth are still lacking [12]. Pectin occupies a central location in the middle lamella (see Figure 17.5) located between the primary cell walls of adjacent cells. Hydrolysis of pectin by enzymes (see Chapter 6) can reduce intercellular adhesion and thus soften the tissue. Nonripening tomato mutants [34] and transgenic tomatoes with drastically reduced polygalacturanase (PG; [14]) demonstrate that PG is necessary for softening to occur during the later stages of ripening. Solubilization of pectin [68] and the release of low-molecular-weight uronides from cell walls occur concomitantly with softening in many fruits. Degradation of hemicellulose may also play an important role in softening. Calcium–pectin interactions also contribute substantially to the rigidity of the cell walls [21]. Calcium salts are frequently added to increase the firmness of processed products like diced tomatoes.

Cellulose is an important component of cell walls, but relatively little is known about its structure within the cell wall. Comparison of nuclear magnetic resonance measurements with sensory evaluation of fruit and vegetable texture suggests that the proportion of crystalline vs. amorphous cellulose in the cell walls determines the relative crispness or toughness [44]. Cellulase gene expression occurs coincident with ethylene-induced fruit softening, but chemical measurements of cellulose have yet to confirm the role of cellulose degradation in softening of fruits [46].

The relative proportion and contents of cell wall constituents vary considerably among species, among cell types, with maturity at harvest, and with elapsed time and storage conditions after harvest. Hydrolysis of these components by carbohydrases produced during ripening along with the removal of calcium leads to softening, while lignification and influx of calcium leads to "hardening."

17.3.1.2.2 Starch and Other Polysaccharides

The principal non-cell-wall carbohydrate of plant tissues is starch, a linear (α-1,4) or branched (α-1,4 and α-1,6) polymer of D-glucose (see Chapter 3). In the developing plant, amylose is synthesized from adenosine diphosphoglucose (ADPG) or uridine diphosphoglucose (UDPG) by the enzyme starch synthetase. This occurs in organelles called amyloplasts. UDPG is formed from sucrose by the enzyme sucrose-UDP glucosyl transferase and ADPG, and ADPG is formed from glucose-1-P by the enzyme ADPG-phosphorylase. A branching enzyme, called Q-enzyme, introduces α-1,6 glucosidic linkages into amylose chains to form amylopectin. Although most starches contain amylose and amylopectin in a ratio of 1:4, high-amylose and high-amylopectin ("waxy") cultivars of cereals have been developed. Waxy starches are valued as industrial products for their bright transparent appearance, which is attributed to the absence of retrogradation. Likewise, short-grain rice is moist, firm, and sticky when cooked due to the high proportion of amylopectin in the starch. In mature plants, starch is localized in intracellular plastids or granules that have species-specific shape, size, and optical properties. Industrially, starch is obtained from such crops as potato, sweetpotato, or cereals that may contain up to 40% starch on a fresh weight basis [53]. Although starch contributes more calories to the normal human diet than any other single substance, it is absent or negligible in most ripe fruits and many vegetables.

Examples of other polysaccharides that occur as intracellular constituents of fruits and vegetables include the water soluble polysaccharide (WSP, phytoglycogen) of sweetcorn, β-glucans of mango, and fructans. D-Fructose in the furanose form is the main unit of fructans and the units are linked β (2→1) or β (2→6). Inulin is the main carbohydrate reserve found in plants that do not accumulate starch (e.g., chicory, Jerusalem artichoke, and sweetpotato). A non-reducing terminus of glucose on inulin indicates that a primer sucrose molecule is built up by successive transfer of fructose units (40–100 residues) from sucrose (Figure 17.13).

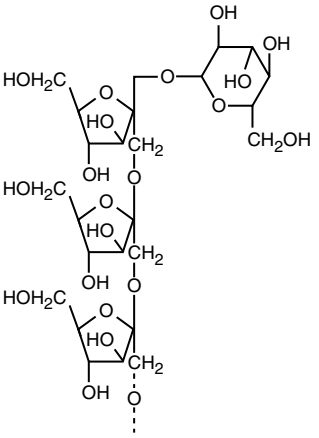


FIGURE 17.13 Inulin, a nonstarch carbohydrate reserve found in some plants. The β (2→1) fructan is the major polysaccharide found in Jerusalem artichoke. The β(2→6) fructans have also been identified in some plants.

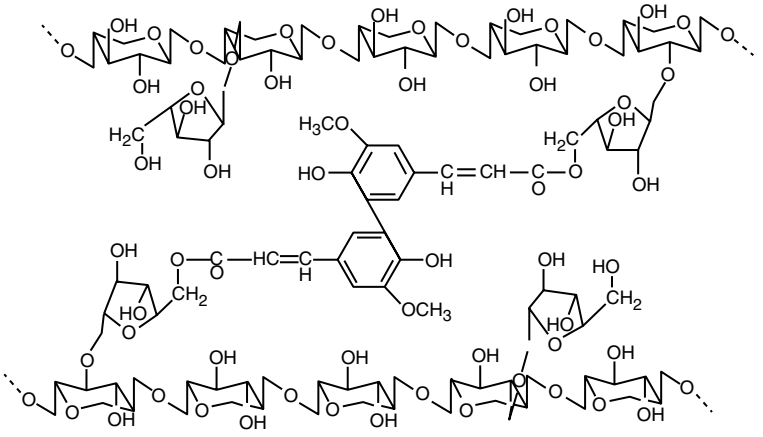


FIGURE 17.14 Portion of a pentosan with ferulic acid cross-link. The arabinoxylan consists of D-xylopyranose units with L-arabinofuranose glycosidic linkages in positions 2 and 3. Covalent cross-links of this type may occur between cell wall proteins and between polysaccharides and proteins.

Oats, barley, and rye are examples of cereals that contain a relatively high percent (5–25% of total carbohydrates) of nonstarch polysaccharides in the flour. The pentosan fraction of cereals is a complex mixture of branched polysaccharides with an arabinoxylan backbone containing small amounts of glucose and ferulic acid (Figure 17.14). Some pentosans are conjugated with protein and they can be separated into water-soluble and water-insoluble fractions. Rye flour contains a relatively high content (6–8%) of pentosans; 15–25% of rye pentosans are water soluble. Pentosans contribute to gluten development in wheat flour and are especially important to the bread-making characteristic of rye flour because of their ability to absorb large amounts of water and to form gels. Insoluble pentosans appear to be formed by peroxidase-catalyzed cross-linking of ferulic acid residues and oxidative gelation. Other nonstarch polysaccharides found in cereal flours are β (1→3) and β (1→4) linear polymers of D-glucopyranose called β-glucans or lichenins. These slimy substances predominate in oats and barley (6–8%), and their high viscosity can cause wort filtration problems in the brewing industry. Glucofructans are also present in significant amounts in durum wheat.

These polysaccharides are branched chain, water soluble, and contain α-D-glucopyranose (1→2), β-D-fructofuranose, β-D-fructofuranose (1→2), and β-D-fructofuranose (6→2) linkages.

Gums and mucilages are also obtained from some plants as tree exudates, seed gums, or as algae extracts (Table 17.12). These carbohydrates are chemically related to the hemicelluloses, and some, such as carrageenan, gum arabic, karaya gum, locust bean gum, guar gum, fuicoidans, laminarins, carob gum, and gum tragacanth, are isolated and used as food additives for stabilization, emulsification, thickening, and gelation (see Chapter 3).

17.3.1.2.3 Simple Sugars and Related Compounds

The sugar content of fruits and vegetables varies from negligible (e.g., avocado) to over 20% (e.g., ripe banana) on a fresh weight basis. Sucrose, glucose, and fructose are the principal sugars of most commodities. In general, fruits and vegetables contain more reducing sugars than sucrose, although in many cases the reverse is true. Other sugars, such a xylose, mannose, arabinose, galactose, maltose, sorbose, octulose, and cellobiose, may also be present, and in some instances they may constitute a major portion of the total sugars. For example, avocado fruit contain significant amounts of heptuloses, heptose, octose, octulose, and nonulose sugars. Branched sugars, such as D-adiose (Figure 17.15), are sometimes constituents of plant glycosides, such as in parsley. Fruit tissues also contain sugar alcohols (alditols), notably sorbitol (Figure 17.15), mannitol, and xylitol. For example, D-sorbitol is present in significant quantities in fruits of the Rosaceae family, and certain mushrooms contain more mannitol than monosaccharides.

TABLE 17.12
Examples of Nonstarch Polysaccharides Obtained from Plants for Use as Food Additives

Polysaccharide	Source	Composition
Gum arabic	Acacia tree exudates	Complex, branched, polymer of D-galactose, L-arabinose, L-rhamnose, and D-galacturonic acid
Carob	Carob tree seeds	Polymannose polymer, β (1→4) with single galactose branch every 4 or 5 mannose residues
Alginates	Brown algae	D-mannuronic acid with β (1→4) bond or L-gluconic acid with α (1→4) bond or copolymer
Carrageenans	Red algae	Linear polymer galactose and sulfated galactose molecules, with β (1→4) or α (1→3) bonds

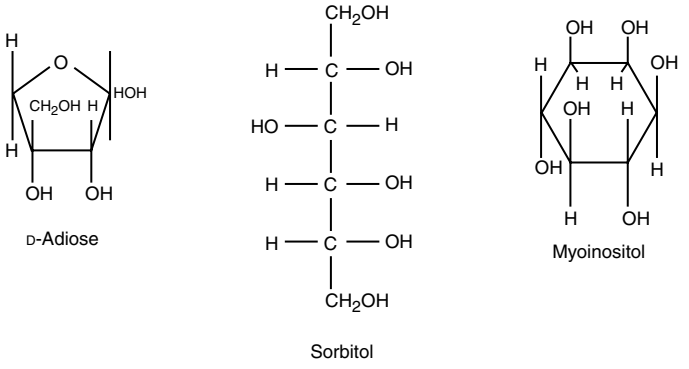


FIGURE 17.15 Examples of monosaccharide derivatives found in some plant tissues.

TABLE 17.13
Percent Storage Protein, Lipid, and Carbohydrate in Various Seed Crops and Tubers

Group	Example	Protein	Carbohydrate	Lipid
High carbohydrate	Wheat	12	80	2.0
	Corn	12	79	6.5
	Rice	9	82	2.0
	Potato	9	82	0.4
	Yam	9	82	1.0
	Cassava	4	85	1.0
High protein	Broad bean	26	57	1.5
	Haricot bean	21	58	1.5
	Lentil	26	56	1.0
	Pea	25	70	2.0
	Chick pea	21	71	5.0
High protein and lipid	Peanut	28	15	50
	Rape (canola)	30	20	40
	Soybean	35	25	21
	Sunflower	35	5	45
	Cotton	0	12	30

Pulses frequently contain significant amounts (1–3%) of raffinose and stachyose, which contain α (1→6) linkages between galactose and glucose. These oligosaccharides are a cause of flatulence when ingested because humans do not produce the α-galactosidase needed to hydrolyze the galactose–glucose bond. Penta- and hexahydroxylic cyclitols are also found in the plant kingdom. Myoinositol (Figure 17.15) is combined with galactose in sugar beet and with phosphoric acid in various edible seeds. The hexaphosphoric ester of myoinositol is called phytic acid. Phytic acid forms complexes with divalent metal ions and reduces the bioavailability of minerals in many grains and legumes (see Chapter 8).

17.3.1.3 Proteins and Other N Compounds

The protein content varies greatly among various fruits and vegetables, although it generally represents only a small percentage of the fresh weight. Certain crops, such as cereals, pulses, tubers, and bulbs, may contain appreciable quantities of protein (Tables 17.11 and 17.13). Proteins are primarily present in plants as enzymes catalyzing metabolic processes. Formation or activation of new enzymes may be physiologically important in various physiological processes (e.g., ripening and senescence). Changes in the levels and activities of enzymes due to changes in cell membrane permeability may be involved in CI.

When appreciable quantities of proteins accumulate, they are normally referred to as "storage proteins," although it is not clear that they are synthesized by the plant for the purpose of storage. These substances may function to lower the osmotic pressure of the amino acid pool, bind ammonia without changing *in situ* acidity, or serve as macromolecular shields to protect other compounds against enzymatic action [62]. Regardless of the role of storage proteins in the physiology or metabolism of the plant, they are very important to the food scientist because of their contribution to nutritive value and functional properties of various crops. Plant storage proteins are generally categorized based on their solubility, but this type of classification is somewhat arbitrary. It should also be recognized that the extraction and separation of plant proteins can be hindered by the presence of tannins and phenolic oxidizing enzymes. A general feature of storage proteins from seeds and tubers

is the presence of nonpeptide amide linkages. Enzymatic deamidation of these proteins can enhance solubility, emulsifying properties, and foaming [24]. Generally, plant storage proteins decrease in molecular weight during senescence. This decrease in molecular weight is partly due to dissociation of larger complexes. Finally, it should be noted that the storage proteins from different cultivars of a given species may exhibit different electrophoretic patterns. Accordingly, electrophoresis or isoelectric focusing can be used to "fingerprint" or identify cultivars of unknown origin [37].

17.3.1.3.1 Nonprotein Nitrogen

Plant tissues may contain appreciable quantities (i.e., 20–75%) of nonprotein nitrogen. For example, more than two-thirds of the nitrogen in potato tuber or apple fruit may be in the form of free amino acids and other constituents. More than 60 different amino acids that are not found in proteins have been identified in plants [8]. Although amino acids are the main nonprotein nitrogen components of most fruits and vegetables, plants may contain appreciable quantities of amines, purines, pyrimidines, nucleosides, betaines, alkaloids, porphyrins, and nonproteinogenic amino acids (Figure 17.16). The contribution of nonprotein nitrogen compounds to quality and postharvest changes is incompletely understood. Some of their roles in fruits and vegetables are known, however. For example, free amino acids probably contribute to the taste of fruits and vegetables, elevated levels of steroidal alkaloids (solanine and chaconine) in some cultivars of potato contribute to bitter taste and toxicity, betanins are red pigments in red beets, and several amino acids and amino acid derivatives are important precursors of aroma compounds in fruits and vegetables.

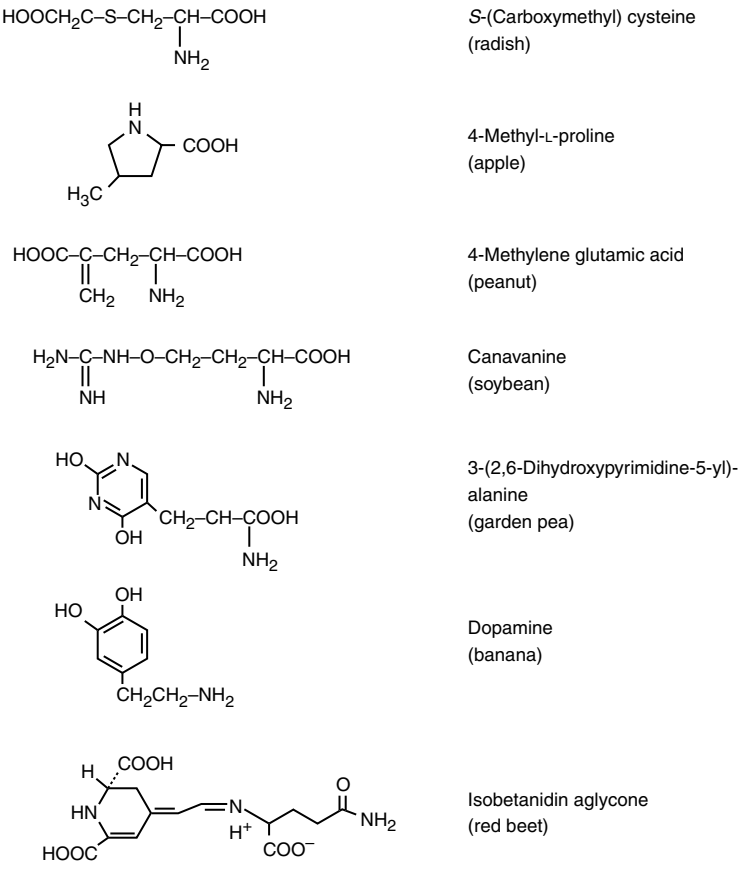


FIGURE 17.16 Examples of nonprotein nitrogen compounds found in edible plants.

TABLE 17.14
Lipid Composition of Wheat Flour

	Fraction Total Flour (mg %)	Nonstarch (mg %)	Starch (mg %)
Triacylglycerols	709	674	35
Diacylglycerols	92	86	6
Free fatty acids	129	110	19
Sterol esters	90	90	18
Glycolipids	582	519	63
Esterified monogalactosyl DG and MG	73	66	7
Esterified steryl glycoside	77	71	6
Monogalactosyl DG	93	87	6
Monogalactosyl MG	30	23	7
Digalactosyl DG	226	214	12
Digalactosyl MG	83	58	25
Phospholipids	1026	242	728
N-Acylphosphatidyl ethanolamine	72	72	
N-Acyllysophosphatidyl ethanolamine	34	34	
Phosphatidyl ethanolamine and phosphatidyl glycerol	19	13	3
Lysophosphatidyl ethanolamine and lysophosphatidyl glycerol	69	10	6
Phosphatidyl choline	104	66	38
Lysophosphatidyl choline	693	36	657
Phosphatidyl serine and related compounds	35	11	24
Total lipids	2628	1703	925

Note: MG, monoacylglycerol; DG, diacylglycerol.

Source: Adapted from Eliasson, A.-C. and K. Larsson. (1993). *Cereals in Breadmaking: A Molecular Colloidal Approach*, Marcel Dekker, New York.

17.3.1.4 Lipids and Related Compounds

Plant lipids are mostly polar, major examples being membrane phospholipids and glycolipids (Table 17.14 and Figure 17.17). Lipids constitute less than 1% of the fresh weight of most fruits and vegetables (Table 17.10) with the exception of those species in which lipids serve as storage reserves (e.g., avocado (4–20%), olive (15–40%), and tree nuts (45–65%)). In most horticultural crops, the lipids that do occur are mainly components of the cell membranes, cuticle, and epidermis. On the other hand, cereals and pulses can accumulate significant amounts of polar lipids, especially in the endosperm. Soybeans contain up to 8% phospholipid. Total oat grain lipid includes 8–17% glycolipids and 10–20% phospholipid. The content of polar lipids is greater in oats than other cereals because they contain a relatively high content of lipid in the endosperm portion of the seed. The polar lipids of wheat flour include several glycolipids and phospholipids in both the starch and nonstarch fractions (Table 17.14). The glycolipids play an important role in gluten development during bread making [16]. Digalactosyl diacylglyceride appears to stabilize the starch–gluten network in wheat flour dough [50].

Oil content is used as an index of avocado maturity and the lipids of the cuticle and epidermis are important to the appearance of most commodities. The cuticle is also important in protection against water loss, pathogens, and mechanical injuries. The increase in respiration rate of climacteric fruit is accompanied by a large increase in phosphatidyl choline and phosphatidic acid, and during the postclimacteric stage there is sudden breakdown (hydrolysis and peroxidation) of total lipids [22].

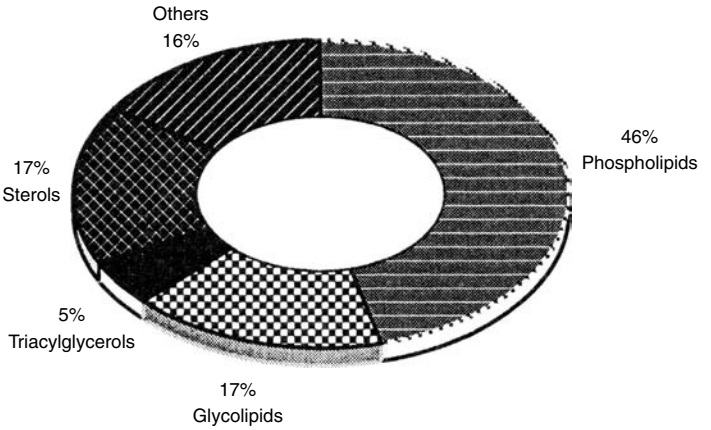


FIGURE 17.17 Lipid fractions of apple flesh (percent of total lipids).

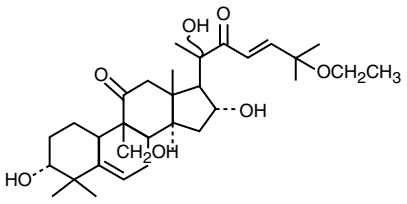
TABLE 17.15
Crude Fat Contents of Rice and Wheat and Their Milling Fractions

Grain Fraction	Crude Fat (% DM)
Rice	
Whole grain (brown rice)	2–4
Milled (endosperm)	<1
Bran	15–22
Embryo	15–24
Polish	9–15
Wheat	
Whole grain	2
Pericarp	1
Aleurone	9
Starch endosperm	1
Germ	10

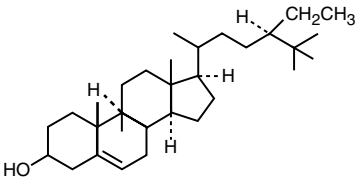
Source: Adapted from Pomeranz, Y. (1992). In *Storage of Cereal Grains and Their Products* (D. B. Sauer, ed.), American Association of Cereal Chemists, St Paul, MN, pp. 55–141.

Membrane lipids may play a role in CI—the degree of fatty acid saturation influences membrane fluidity and may change upon exposure to chilling temperature. Chilling-sensitive plants tend to have a high percentage of saturated fatty acids especially in the phosphatidylglycerol pool. Such membranes can undergo a phase change at chilling temperatures.

Triacylglycerols of the neutral lipid fraction are the main reserve material in plant tissues such as nuts, oil seeds, pulses, and cereals (Tables 17.3 and 17.5). Reserve triacylglycerols occur mainly as lipid droplets (<10 μm) in the germ (embryo and associated tissue) and bran (aleurone surrounding endosperm) portions of the seed. The crude fat content of milling fractions from two cereals is shown in Table 17.15. Neutral fats account for 85–90% of rice bran oil and 60% of rice endosperm lipids. In wheat, neutral lipids predominate in the germ (80%) and aleurone (72%), while polar



Curbitacin (cucumber)



Sitosterol (24-α-ethyl cholesterol)
(common plant steroid)

FIGURE 17.18 Examples of triterpenoids found in the total lipid fraction of plants.

lipids predominate in the nonstarch endosperm (67%) and starch endosperm (95%) fractions. Corn oil consists of about 95% triacylglycerols with 1.5% phospholipids, 1.7% free fatty acids, and 1.2% sterols. The major fatty acids in cereal grain and pulse lipids are linoleic ($C_{18:2}$), oleic ($C_{18:1}$), and palmitic (C_{16}). Palm and copra seed oils are unique in containing a high percentage of lauric (C_{12}) and myristic (C_{14}) along with palmitic and oleic acids. Cocoa fat contains a high percentage of stearic along with palmitic and oleic acids. The distribution of principal fatty acids in various vegetable oils is presented in Chapter 4.

17.3.1.4.1 Other Lipid Substances

Unsaponifiable lipids in seed oil include sterols, higher alcohols, and hydrocarbons like squalene. Several milligrams of carotenoids per 100 g of fresh weight are present in some fruits (e.g., pineapple, cantaloupe, and orange) and vegetables (e.g., tomato, red pepper, and carrot). Different kinds of carotenoids accumulate in plant crops (discussed in Chapter 9). During plant senescence chloroplast carotenoids are degraded while chromoplast carotenoids are synthesized. Bitter tasting triterpenoids occur in the seeds and flesh of Rutaceae fruits (limonin) and Cucurbitaceae fruits (curcurbitacins) (Figure 17.18). Phytosterols include compounds that are structurally similar to cholesterol such as sitosterol, campesterol, and stigmasterol (Figure 17.18). The ratio of phytosterols to other sterols is characteristic of plant species and has been used to detect adulteration of plant oils.

Lipid substances are also prominent in the protective epidermal cells of certain plant organs. Some examples of these protective substances are illustrated in Figure 17.19. Waxes that protect the surfaces of leaves and seeds from dehydration include esters of long-chain alcohols and fatty acids. The waxy cuticle consists of a complex polymer of hydroxyl fatty acids called cutin.

Other lipid or lipophilic substances, although present in trace quantities, may contribute to the characteristic flavor of edible crops. Odoriferous substances of fruits are mainly oxygenated compounds (esters, alcohols, acids, aldehydes, and ketones), many of which are derivatives of terpenoid hydrocarbons or lower aliphatic acids and alcohols. The range of such volatile constituents present in vegetables is generally more limited than that found in fruits. In some instances, these substances are dissolved in terpenoid hydrocarbons and localized in special oil sacs (e.g., essential oils of peppermint leaf). In other cases, the components are formed as a result of cellular decompartmentation during wounding, cooking, or chewing the tissue.

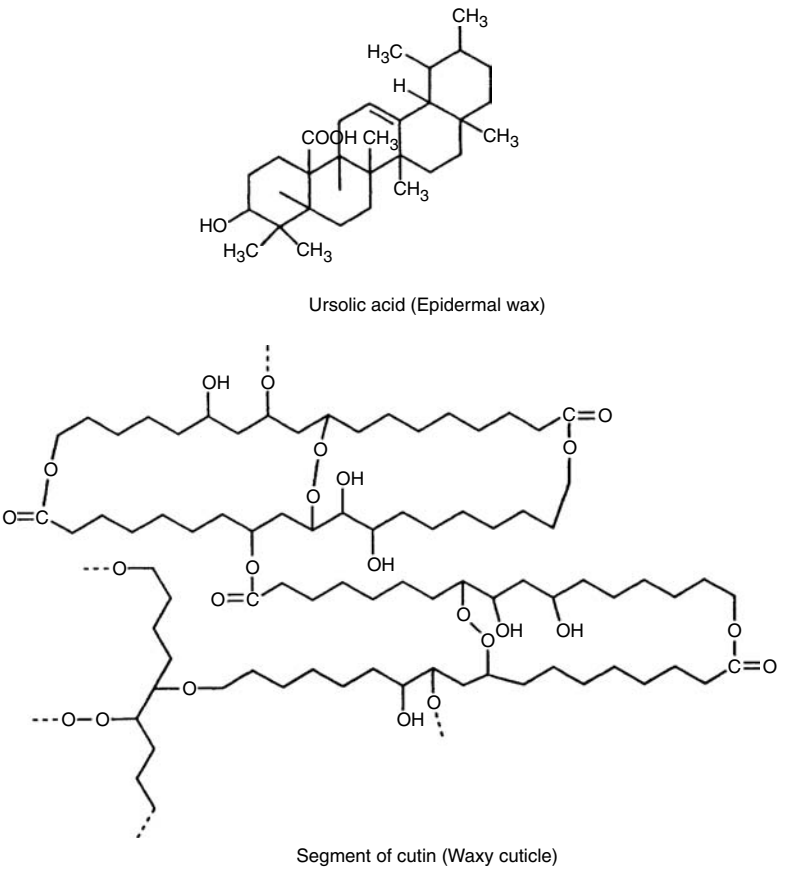


FIGURE 17.19 Waxy substances associated with plant epidermal cells.

17.3.1.5 Organic Acids

Organic acids are important in respiratory metabolism and as storage compounds in fruits and vegetables. Small quantities of organic acids occur in plants as metabolic intermediates (e.g., in the tricarboxylic acid or Krebs cycle, glyoxylate cycle, or shikimic acid pathway) and they may also accumulate in vacuoles. The Krebs cycle is the main channel for the oxidation of organic acids in living cells and it provides the energy required for maintenance of cell integrity. The accumulation of organic acids gives rise to an acidic or sour taste. Most fresh fruits and vegetables are acidic, but fruits are generally more acidic than vegetables. Acid levels range from very low (e.g., sweetcorn) to high in such crops as currant, cranberry, or spinach. Some fruits, such as lemons and limes, contain as much as 2–3% acid of their total fresh weight. Titratable acidity, specific organic acids present and their relative quantities, and other factors influencing the buffering system affect pH, which can vary from 2 to 7 among various commodities. An acidic crop may contain over 50 mEq acid per 100 g of tissue.

17.3.1.5.1 Aliphatic Plant Acids

The most widely occurring and most abundant acids in plants are citric and malic (Table 17.16 and Figure 17.20) each of which can constitute up to 3% of the tissue on a fresh weight basis. Instances in which neither malic nor citric acids predominate include grapes and avocado (tartaric), spinach (oxalic), and blackberry (isocitric). The total acidity of fruits usually decreases during ripening due to use of organic acids in respiration or their conversion to sugars, although specific acids may actually increase.

TABLE 17.16
Predominant Organic Acids in Various Fruits and Vegetables

Predominant Acid	Crop
Citric	Lemon, orange, currant, fig, gooseberry, guava, loganberry, pineapple, pomegranate, raspberry, strawberry
	Leafy vegetables, legumes, tomato, potato, sweetpotato
Isocitric	Blackberry
Malic	Apple, apricot, banana, cherry, grape (about equal to tartaric), peach, pear, plum
	Artichoke, broccoli, carrot, cauliflower, celery, cucurbits, lettuce, okra, onion
Oxalic	Spinach
Quinic	Kiwifruit
Tartaric	Avocado, grape (about equal to malic)

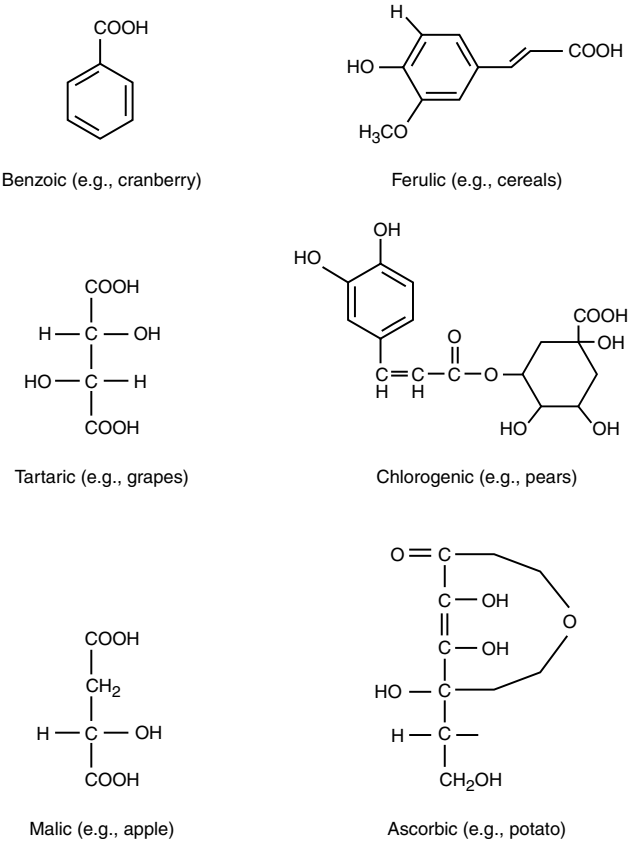


FIGURE 17.20 Examples of organic acids in plant tissues.

17.3.1.5.2 Carbocyclic Plant Acids

Various aromatic acids are also found in plant tissues (Figure 17.20). The alicyclic acids, quinic (kiwifruit) and shikimic, are widely distributed in the plant kingdom as key intermediary metabolites. Some acids, such as chlorogenic, are important food constituents because of their contribution to both enzymatic and nonenzymatic browning reactions. Ferulic acid is involved with cross-linking of pentosans in cereal flours (Figure 17.14). Others, such as benzoic, are important antifungal agents in crops such as cranberry.

17.3.1.6 Phenolic Acids

Phenolic compounds in plant foods include a wide range of compounds and a broad spectrum of functional activities. Traditionally, these compounds have been considered important in plant foods because of their impact on flavor and color (particularly enzymatic browning; see Chapter 6) but there is substantial current interest in their potential health benefits [27], antioxidant activity ([52]; see Chapter 12), and antimicrobial [17] effects. Phenolics are thought to play a role in the resistance of some immature fruit tissues to attack by pathogens.

In the simplest chemical terms, phenolic compounds include a hydroxylated aromatic ring such as phenol, *p*-cresol, and 3-ethylphenol. Phenolic acids such as caffeic, coumaric, and ferulic occur widely in the shikimic acid pathway of plant tissues, which begins with the condensation of phosphoenolpyruvate and erythrose-4-phosphate (Figure 17.21). These compounds often occur as esters with sugars or other phenolics, or as a part of tannins. Chlorogenic acid is the ester of caffeic and quinic acids, occurs very widely, and is the major phenolic substance in apples and pears [61]. Phenylalanine and tyrosine are synthesized in the shikimic acid pathway and they can serve as important precursors for the formation of phenolic acids, the latter resulting in increased lignin biosynthesis and enzymatic browning.

Most phenolic compounds can serve as substrates for enzymatic browning, and they can also contribute to darkening by forming complexes with metal ions such as copper and iron. Sequestrants like ethylenediamine tetraacetic acid (EDTA) or phosphates are frequently used to reduce the formation of these undesirable metal–phenol complexes. Phenolics are the main substrates of enzymatic browning reactions of cut or damaged tissues of apple, peach, potato, and so forth upon exposure to air. Differences in rates and extent of browning in different plant tissues are attributable to several factors, including availability of substrate (oxygen or the phenol), enzyme activity, compartmentation of enzymes and substrates, and available metal ions. Changes in phenolic substrates during ripening and storage can affect browning in a given tissue.

Phenolic content is generally higher in fruits than vegetables and is higher in immature than mature fruits. Fruits typically show a decline in phenolic compounds with ripening, and an increase in response to stresses such as bruising and fungal infection. The extent and magnitude of these changes varies widely depending on the plant material and the conditions of storage. Shredded carrots accumulate *trans*-5′-caffeolyquinic acid rapidly when stored in air, but this is inhibited by high concentrations of carbon dioxide or low concentrations of oxygen [3]. Similarly, shredded lettuce accumulates phenolics more slowly when stored in the type of atmosphere just mentioned [40].

17.3.1.6.1 Tannins (Polyphenolics)

Polyphenolics have traditionally been separated into "condensed" and "hydrolyzable" tannins. These terms are somewhat confusing because both groups can be hydrolyzed. Condensed tannins are more correctly referred to as flavan-3,4-diol-derived tannins or proanthocyanidins, and the hydrolyzable tannins as gallotannins or ellagitannins (Figure 17.22). Polyphenolics provide a number of different functionalities in foods, including color and astringency. Astringency in immature (i.e., "green") fruits is related to the content of tannins. Tannins are a diverse group of molecules that range up to 3000 Da and are formed from carbocyclic acids, phenolic acids, and sugars. The exact structures of the larger molecules are not known.

The name tannin comes from the use of plant extracts to treat hides to make leather. Tannin–protein interactions are also important in foods like beer where the tannins from the hops react with protein to form "haze." Formation of tannin–protein complexes has been thought to reduce the nutritive value of tissues with high-tannin content [57], but the antinutritional effects may actually be caused by a more direct physiological effect of the tannins [13]. Tannin–protein complexes involve both hydrogen bonds and hydrophobic interactions and may generate covalent links through amide and sulfhydryl groups when the phenolics are oxidized [25]. Proteins with high-proline content have a greater affinity for tannins than do other proteins. The astringency of many fruits is thought to be

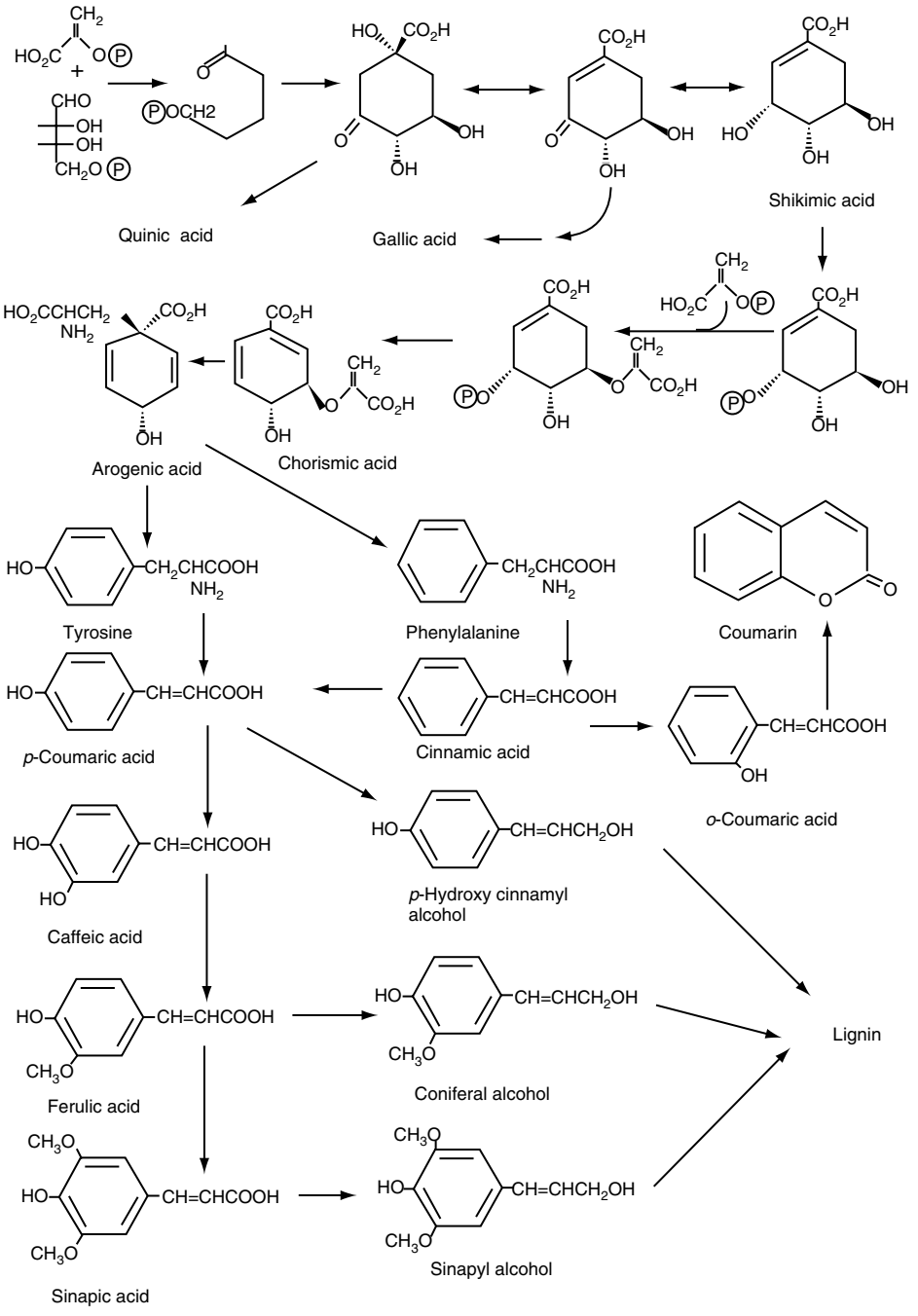


FIGURE 17.21 Biosynthesis of some phenolic acids (shikimic acid pathway).

caused by tannins interacting with the mucous membranes of the mouth. Tannins also form complexes with caffeine resulting in the formation of a precipitate known as "tea cream" [51]. Gelatin, because of its amino acid composition, can be used to complex tannins and reduce the astringency of wines.

Tea is the most widely consumed beverage in the world, and many of its characteristics are related to tannin content [4]. When converting freshly picked tea leaves into tea, the major concern

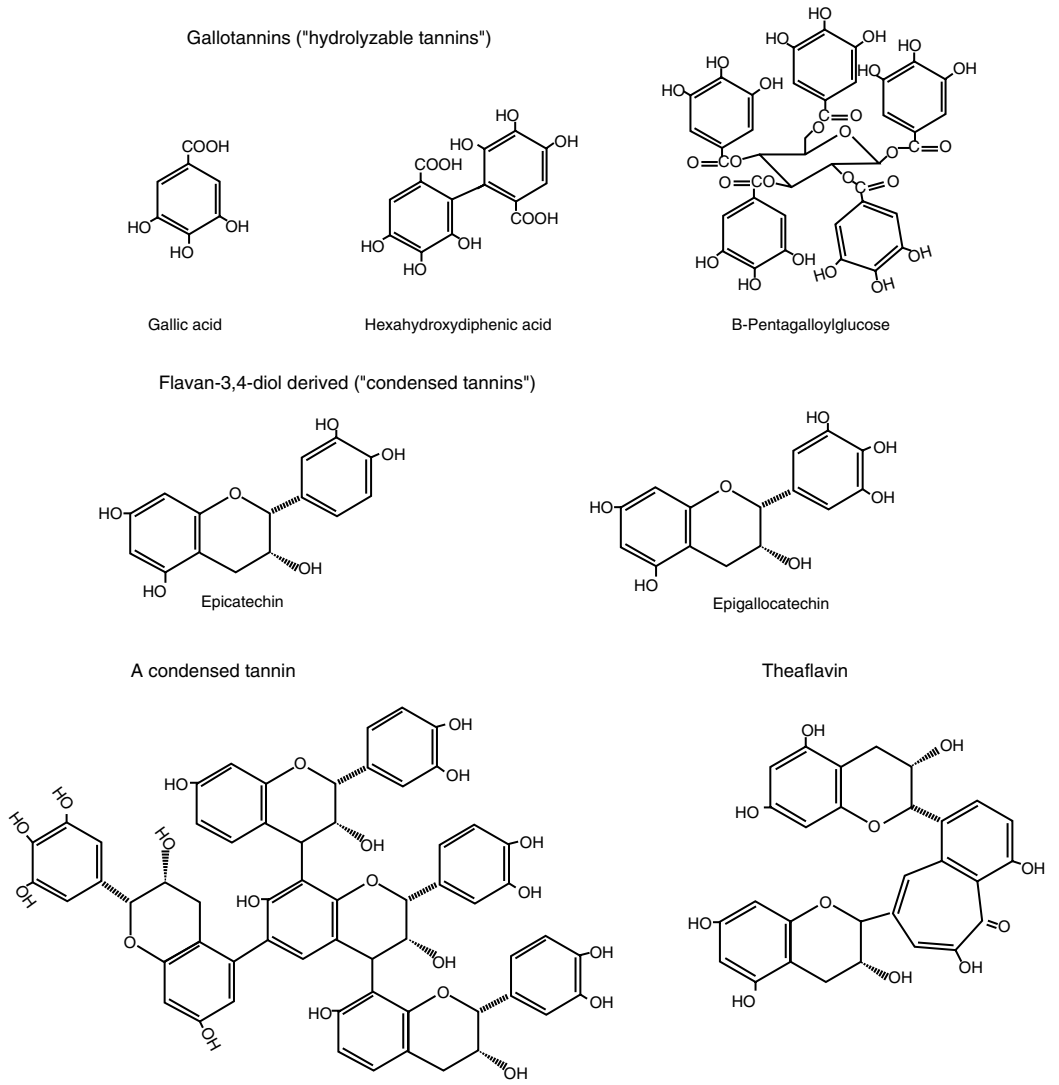


FIGURE 17.22 Structure of tannins found in plant tissues and products.

is controlling oxidation of polyphenols. For green teas, the leaves are blanched at the beginning of the process to reduce oxidation of polyphenols. Production of black teas on the other hand requires oxidation of polyphenols, and thus the processing steps are designed to enhance and control their enzymatic oxidation. The processing sequence includes reducing the moisture content of the leaves to concentrate the phenols (withering), followed by a crushing step that breaks down the cellular compartmentation and allows access of oxidizing enzymes to substrates. After the desired degree of oxidation, the process is terminated by blanching. For oolong tea (mildly oxidized) the oxidation process is stopped soon after crushing.

Fresh green tea leaves contain about 40% (dry basis) polyphenols with epigallocatechin gallate, epigallocatechin, and gallocatechin being the major types. Oxidation of catechins and gallotannins with subsequent condensation leads to the formation of the flavins, which are responsible for the reddish color of black teas. Epitheaflavic acids arise similarly from epicatechins and gallic acid. The arubigens are a complex group of polymeric procyanidins that are responsible for the brown color

of tea. There is substantial interest in the antioxidant properties and health effects of polyphenolics (see Chapter 12).

17.3.1.7 Pigments

The principal pigments of plant tissues—the chlorophylls, carotenoids, and flavonoids—are discussed in Chapter 9. The extent of pigment synthesis and degradation in detached fruits and vegetables can be influenced by storage conditions, such as light, temperature, and relative humidity, and by volatile substances such as ethylene. Ethylene is formed in plant tissue during ripening, following wounding, or by exposure of the plant to air pollutants. By virtue of its hormone action, ethylene initiates the degradation of chlorophyll in mature plant tissues such as fruits and leafy vegetables. Normally, we wish to retard the process in vegetables (via low temperature and MA storage) and promote it in ripening fruit (by ethylene exposure: "degreening"). The exact mechanism by which ethylene action leads to chlorophyll degradation is not completely known. Chlorophyllase (EC 3.1.1.14) is a thylakoid membrane glycoprotein that catalyzes hydrolysis of the phytol side chain of chlorophyll (no change in color), although it appears that hydrolysis is followed by oxidative degradation of the tetrapyrrole involving a peroxidase (chlorophyll:H_2O_2 oxidoreductase). The participation of different lipoxygenase isoenzymes has also been implicated in chlorophyll and carotenoid degradation in growing plants and seeds [33].

Carotenoids (yellow, orange, and orange–red pigments) are very stable compounds that remain intact even when senescence is well advanced. Content of β-carotene (provitamin A), a major carotenoid, is important for nutrition. Synthesis of carotenoids is important during fruit development, but may be masked by chlorophyll (e.g., citrus, bananas). In ripening tomato, carotenoid synthesis is concurrent with chlorophyll degradation. The transformation of (green) chloroplast to (yellow-to-red) chromoplast is a major metabolic event in ripening fruit. In citrus fruits, this transformation appears to be influenced by day–night temperatures (cool nights promote chloroplast–chromoplast transformation) and also the balance of nitrogen and sucrose in the peel tissue [28].

Anthocyanins (red, blue, and purple pigments) are flavonoids—water soluble, acid-stable, glycosidic phenolic compounds that are readily hydrolyzed to free anthocyanidin or oxidized to give brown oxidation products. The colors of anthocyanins are influenced by vacuolar pH. Often they are confined to the cells of the epidermal layer of fruits, in which case the color is referred to as "blush."

17.3.1.8 Vitamins

Plant tissues are an important source of several vitamins that are essential in human nutrition. The contribution of edible plant tissues to the dietary intake of some vitamins is summarized in Table 17.11. The vitamin content of a given species can vary considerably with variety, growing conditions, postharvest storage conditions, and processing. For example, the vitamin C content of spinach varies from 40 to 155 mg/l00 g fresh weight. Oilseeds, notably soybean, are rich in vitamin E (>10 mg/100 g fresh weight). Some fruits and vegetables are a particularly rich source of vitamin C (e.g., guava, black currant, strawberry, and kale) and provitamin A (e.g., lettuce, spinach, and carrots). Cereal grains are very good dietary sources of vitamins B_1, B_2, and B_6.

Vitamins are usually distributed nonuniformly in plant tissues. The old adage, "It's a sin to eat the potato and throw away the skin," makes reference to the greater concentrations of thiamin and ascorbic acid in the cortex of the tuber as compared with the interior. Separation of the starchy endosperm from the germ and aleurone during milling of cereals results in substantial loss of B vitamins and minerals.

The water-soluble vitamins, especially ascorbic acid, are very susceptible to postharvest degradation when fruits and vegetables are exposed to adverse handling and storage conditions. Ascorbic acid losses are minimized when fruits and vegetables are handled to avoid physical injuries, water loss (wilting), delays before cooling, and CI. Low O_2 concentrations in the storage atmosphere also

TABLE 17.17
Mineral Composition of Major Elements in Some Edible Plants

	Mineral (mg/100 g Fresh Weight)							
Crop	**K**	**P**	**Mg**	**Ca**	**Na**	**Fe**	**Cl**	**Zn**
Pulses								
Soybean	1400	490	210	210	6	7	6	1
Peanut	710	370	160	59	5	2	7	3
Vegetables								
Red beet	336	46	1	29	86	1		1
Tomato	297	26	20	14	6	1	60	
Green beans	256	37	26	51	2	1	36	
Cereals								
Wheat	454	433	183	45				

reduce ascorbic acid losses. Postharvest losses in vitamins A and B, while usually much smaller than losses in vitamin C, can occur at high temperatures in the presence of oxygen.

17.3.1.9 Mineral Elements

The total mineral content of plant tissues is sometimes expressed as ash content (residue remaining after incineration). The ash content of plant tissue varies from less than 0.1% (e.g., yams) to over 4% of the fresh weight (e.g., kohlrabi). Mineral content is influenced by species characteristics as well as by agronomic practices. The mineral composition of some edible plant tissues is shown in Table 17.17 and the contribution of plant foods to dietary intake is shown in Table 17.11. The most abundant mineral elements in plants are potassium, calcium, magnesium, iron, phosphorus, sulfur, and nitrogen. Potassium is the single most abundant element in most plants; for example, parsley contains over 1% on a fresh weight basis. Calcium is the second most important mineral constituent and is mainly associated with cell walls and membranes. Magnesium is a component of the chlorophyll molecule, while phosphorus is a constituent of proteins that are important in carbohydrate metabolism and energy transfer.

The distribution of particular minerals in a given tissue is known to be nonuniform. In peas, phosphorus is many times richer in cotyledons than in testa, whereas the difference is reversed for calcium. Mineral elements occur mainly as salts of organic acids. During storage of fruits and vegetables, active exchange of minerals occurs between different physiologically active centers (e.g., stem, inflorescence, storage organs, and leaves) [72].

The mineral content of a given species can influence physiological disorders that arise pre- and postharvest. For example, preharvest calcium deficiency is the cause of numerous disorders, the symptoms of which typically appear during postharvest storage, while if calcium is increased by agronomic practices or by postharvest treatments, this can result in improved storage life and product quality.

17.3.2 Compositional Changes That Influence Plant Food Quality

Studies of the various compositional changes in plant organs during their developmental stages are essential to determining their optimum horticultural (harvest) maturity. Such information is also important in relating sensory characteristics to composition of the commodity and in developing

means of controlling the rate of compositional changes. The physiological orchestration of chemical change at the cellular level involves several strategies. These include biological clocks and environmental factors that elicit gene expression, metabolic feedback and feed-forward sites in pathways, changes in cell compartmentation of reactants, and inhibitors and activators of enzyme-catalyzed reactions. In addition to cellular control mechanisms, factors such as temperature, oxygen concentration, pH, and cellular integrity can have an important influence on postharvest chemical changes in both fresh and processed plant tissues. Quantifying differences in compositional changes as influenced by the postharvest environment is important in selecting optimum conditions that would result in the best possible quality for the consumer.

Chemical changes in postharvest fruits and vegetables are subject to a wide array of biochemical control mechanisms. The mere presence of an enzyme does not alone indicate its functions in association with a physiological event. For example, invertase is present in potato tubers, but its activity is sometimes arrested by a proteinaceous invertase inhibitor. Flavor precursors are not necessarily converted to flavor compounds *in situ*, despite the presence of appropriate "flavorases," since the reactants may be physically separated within the cell microstructure. The importance of cell disruption in the biochemistry of processed fruits and vegetables cannot be over emphasized [58]. In particular, cell disruption influences the formation of flavors, off-flavors, and browning reactions.

Subtle differences in microconstituents, such as inorganic ions, can also profoundly influence the direction of metabolism by modifying the activity of a particular enzyme. For example, calcium ions appear to influence association–dissociation of peroxidase from cell walls and thereby limit peroxidase-catalyzed reactions associated with lignification of cell walls. Control factors for enzyme-catalyzed reactions are discussed in more detail in Chapter 6.

17.3.2.1 Changes in Cell Wall Constituents

Because the cell wall is the primary structural element in plant tissues, it is central to the textural quality of plant foods. In some cases, such as apples, celery, and lettuce, crispness is desired, while in others, such as peaches, strawberries, and tomatoes, a soft texture is preferred. Changes in the cell wall have a major influence on softening of plant tissues. Softening may also occur from a lack of turgor, but in most fruits and vegetables softening is the result of changes in cell wall polysaccharides caused by depolymerization, demethylation, or loss of calcium. Hardening may also occur as a result of lignin formation in the wall; examples include asparagus, celery, and pears. In asparagus and celery, walls of vascular tissue are the primary sites for lignin synthesis, whereas in pears it occurs mostly in the stone cells.

Softening is generally accompanied by solubilization of pectin and the release of polyuronides. Pectin methylesterase (PME), which catalyzes removal of the methyl ester from pectin, occurs widely in plants and fungi. In the case of tomato fruit 90% of the pectin is methylated in mature green fruit, and as PME increases during ripening, this declines to about 30% in ripe fruit. PME in banana also increases with ripening. In apple, guava, mango, and strawberry, however, PME decreases during ripening. Endo-PGs, which would be expected to have a greater effect on pectin structure than the exo-PGs (see Chapter 6), are not found universally in fruits and are notably absent in apples [6]. Apples do contain exo-PGs. In most fruits, PG and cellulase increase during ripening. PG and PME increase during ripening of tomato fruit, but this does not totally account for the softening that occurs. Hemicellulases and cellulases are also present during ripening and are thought to play an important role in softening. Although most of the information on cell wall changes during ripening is related to degradation, it must be noted that some synthesis also occurs [41].

The amounts and types of pectic enzymes in fruit have an important bearing on yield, clarity, and cloud stability of fruit juices and other beverages derived from them. In citrus and tomato juices, in which a stable colloidal suspension is desired, the presence of PMEs and PGs is undesirable. In citrus juices, obtaining a stable cloud is made more difficult because one form of the PME is quite stable to heat [73]. The classic "hot break" process for tomato juice is based on inactivating PME

before cellular disruption because the latter would otherwise result in substantial de-esterification of pectin. PG is added in some processes to solubilize pectin and increase the yield of soluble solids.

Moderate heating, for example, at 60–70°C, of tissues causes disruption of membranes and decompartmentation of cell constituents. For some vegetables (e.g., potatoes and green beans) mild heating leads to firming of texture. Heat-induced firming is believed to start with damage to cell membranes that causes an increase in permeability. This leads to (1) liberation of Ca^{2+} (e.g., from starch phosphate in potato) and its diffusion to the cell wall/middle lamella, (2) activation of PME and de-esterification of pectin, and (3) formation of Ca^{2+} (and/or Mg^{2+}) ionic cross-linkages between carboxyl groups of pectin. Ca^{2+} redistribution may also be involved in the "hard to cook" problem of legume seeds. It is thought that extended storage of beans at high temperature and relative humidity may allow phytase to catalyze conversion of calcium phytate to myoinositol and allow the migration of free Ca^{2+} to the middle lamella, where it causes cross-linkages to form. Postprocess softening of canned apricots is caused by chelation of structural calcium from the fruit cell wall by organic acids, notably citric acid [18].

17.3.2.2 Starch–Sugar Transformations

Synthesis of starch and its degradation to simple sugars are important metabolic events in postharvest commodities. The presence of starch degrading enzymes in plant tissues can cause loss of viscosity in formulated food products that have been thickened using starch. In potatoes, sugars are undesirable since they can cause poor texture after cooking, an undesirable sweet taste, and/or excessive browning during frying. Conversion of starch to sucrose and reducing sugars is stimulated 5- to 20-fold in most potato cultivars when they are stored at nonfreezing temperatures below 5°C. Current evidence indicates that biosynthesis of sucrose, catalyzed by sucrose synthase (Figure 17.23), at low temperature serves to utilize hexose-6-phosphates (via fructose-6-P), which would otherwise cause feedback inhibition of glycolysis. Glucose-6-phosphate is an inhibitor of phosphorylase-catalyzed degradation of starch. At a storage temperature above 10°C, sucrose, or perhaps sucrose phosphate, functions to limit starch hydrolysis via feedback control on phosphorylase or by promotion of starch synthesis via ADP glucose–starch glucosyltranferase. In developing potato tubers, phosphorylase catalyzes synthesis of α-1,4-glycosidic linkages, whereas in mature tubers it functions primarily to catalyze starch breakdown. Amylases, which are important in starch catabolism in germinating tubers, do not appear to be very active in the dormant tuber. Potato tubers contain a protein inhibitor of the enzyme invertase (Figure 17.23) that becomes inactive at low, nonfreezing temperatures (cold stress). Thus sucrose is partially converted to fructose and glucose. Theories to explain the reason for cold sweetening in potato cultivars include the cold lability of phosphofructokinase and possibly pyruvate kinase [2], bypassing phosphofructokinase via the pentose phosphate cycle [69], and loss of starch granule integrity [5].

Inhibition of starch synthesis at low temperature may also play a role in cold sweetening. Blocking the expression of ADP-glucose pyrophosphorylase (ADPGP) with an antisense gene in transgenic potatoes results in the abolition of starch formation and massive accumulation of sucrose (30% of dry weight) [42]. ADPGP catalyzes formation of the nucleotide sugar (ADPG) that is incorporated into the polyglucan chain during amylose synthesis:

$$\text{ADPG-pyrophosphorylase}$$

$$\text{Glucose-1-phosphate} + \text{ATP} \rightarrow \text{ADPG} + \text{PP}_i$$

$$\text{ADPG–starch synthetase}$$

$$\text{ADPG} + (\text{Glucose})_n \rightarrow (\text{Glucose})_{n+1} + \text{ADP}$$

Blocking the expression of ADPGP also results in a major increase in the mRNA for the enzyme sucrose phosphate synthase.

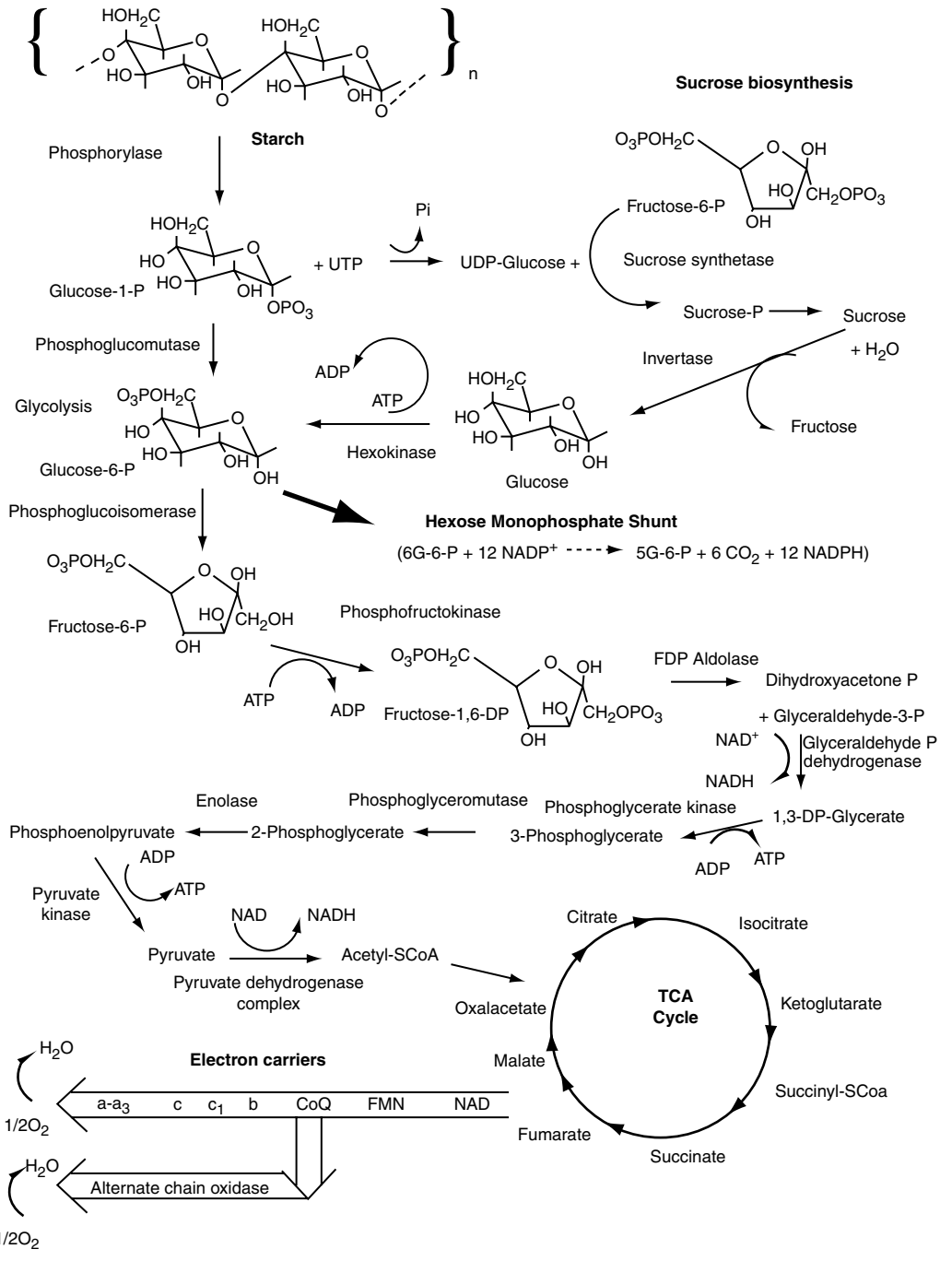


FIGURE 17.23 Respiratory metabolism of starch and sucrose in plants. The glycolytic pathway converts 1 mole of glucose or glucose-1-phosphate to 2 mole of pyruvate. The hexose monophosphate shunt reduces 12 mole of NADP by oxidation of 1 mole of glucose-6-phosphate to carbon dioxide. The tricarboxylic acid cycle results in the conversion of pyruvate to carbon dioxide, with the formation of ATP, NADH, and $FADH_2$. The electron transport system links the oxidation of NADPH and $FADH_2$ with the reduction of molecular oxygen to water and the synthesis of ATP from ADP and P_i at three phosphporylation sites. The alternate electron transport system branches from the electron transport system at ubiquinone (CoQ) and is not coupled to oxidative phosphorylation of ADP to ATP.

Regardless of how sugars accumulate during cold storage of plant commodities, this does represent a problem during commercial storage of potatoes. In practical situations, this problem is overcome by "reconditioning" (holding for several days at warm temperatures to reduce the sugar content through catabolism and conversion to starch) or by leaching out unwanted sugar by exposure of cut potatoes to water.

In some commodities, notably physiologically immature seeds (peas, sweetcorn, and green beans), synthesis of starch, rather than degradation, may predominate after harvest. Starch synthesis is generally optimal at temperatures above ambient. Diminution of sugars accompanies starch synthesis in such crops and is normally detrimental to quality. For example, sucrose may serve as a substrate for sugar nucleotides used in starch synthesis as follows:

$$\text{Sucrose} + \text{UDP} \xrightarrow{\text{Sucrose-UDPG glucosyltransferase}} \text{UDP-glucose} + \text{fructose-1-phosphate}$$

$$\text{Fructose-1-phosphate} \xrightarrow{\text{Phosphoglucoisomerase, Phosphoglucomutase}} \text{Glucose-1-phosphate}$$

$$\text{Glucose-1-phosphate} + \text{ATP} \xrightarrow{\text{ADPG-pyrophosphorylase}} \text{ADPG} + \text{PP}_i$$

The principal enzymes in starch synthesis appear to be those in photosynthetic tissues, namely, fructose transglycosidase, ADPG:starch glucosyltransferase, UDPG pyrophosphorylase, sucrose–UDPG glucosyl transferase, and ADPG pyrophosphorylase. ADPGP is inhibited by pyrophosphate, and sprays containing these substances are sometimes applied to sweetcorn to retard the conversion of sugar to starch [1]. Decreases in sugars may also result from their oxidation via mitochondria-linked reactions. Details of starch synthesis in postharvest commodities are not completely understood.

17.3.2.3 Metabolism of Organic Acids

Organic acids are in a constant state of flux in postharvest plant tissues and tend to diminish during senescence. Much of the loss is attributable to their oxidation in respiratory metabolism as suggested by the increase in RQ (see Section 17.2.2). The RQ is approximately 1.0 when sugars are substrates, increases to 1.3 when malate or citrate are substrates, and further increases to 1.6 when tartrate is the substrate. Some suggested pathways associating respiratory metabolism with organic acid metabolism are shown in Figure 17.24. Certain enzymes (e.g., malic enzyme and pyruvate decarboxylase) and the RQ increase concomitantly with climacteric respiration of certain fruits.

The metabolism of aromatic organic acids via the shikimic acid pathway (Figure 17.25) is important because of its relationship to protein metabolism (aromatic amino acids), accumulation of precursors of enzymic browning (e.g., chlorogenic acid), and lignin deposition (phenyl propane residues). It should also be mentioned that acetyl coenzyme A (acetyl-CoA) participates in the synthesis of phenolic compounds, lipids, and volatile flavor substances. Apart from their general importance to flavor, texture, and color, certain organic acids appear to modify the action of plant hormones and are thereby implicated in control of ontogenic changes. For example, the finding that chlorogenic acid modifies the activity of indole-3-acetic acid oxidase may well relate to the increased concentration of this acid in the green blotchy areas of defective tomatoes. The biosynthesis of flavor substances in fruits and vegetables sometimes involves organic acids. For example, the formation of isoamyl alcohol and glutamic acid in ripening tomato involves the enzyme L-leucine:2-ketoglutarate amino transferase (Figure 17.26).

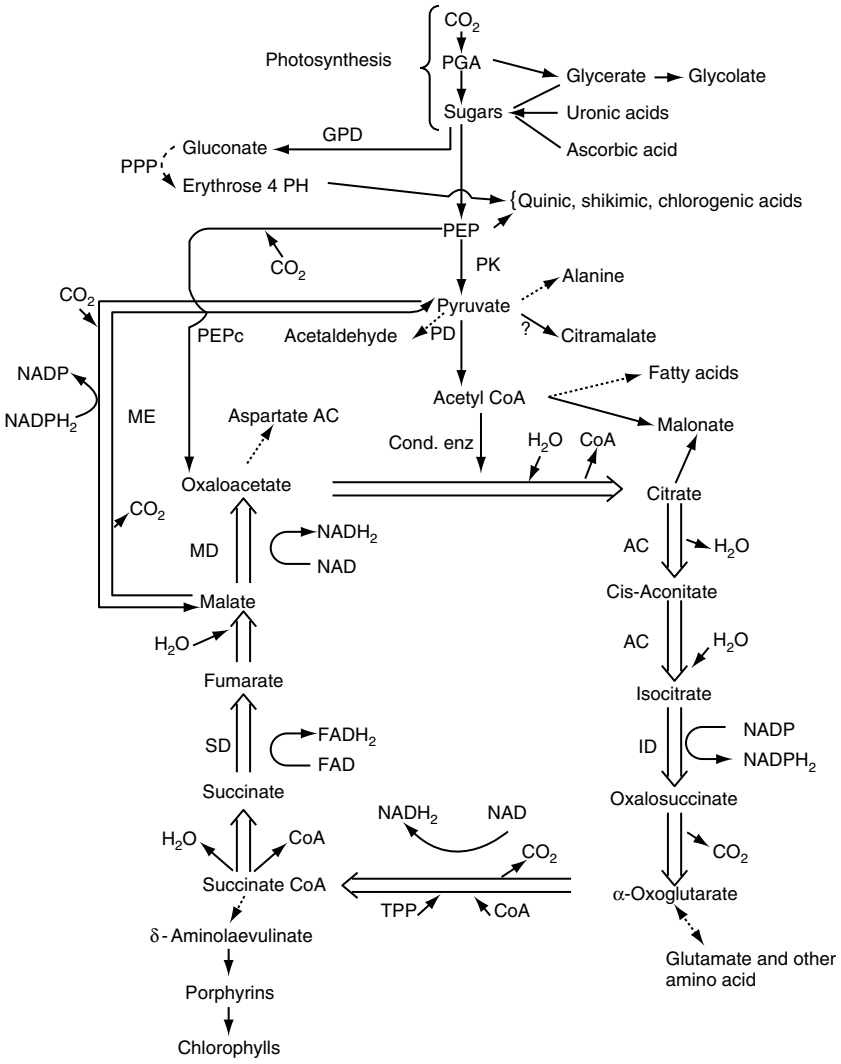


FIGURE 17.24 Krebs cycle (=>) with some other modes of organic acid synthesis (→) or synthesis of other important constituents (→). Abbreviation: AC, aconitase; Cond. enz., condensing enzyme; GPD, glucose-6-phosphate dehydrogenase; ID, isocitric dehydrogenase; MD, malate dehydrogenase; ME, malic enzyme; PEPc, phosphoenolpyruvate carboxykinase; PGA, phosphoglyceric acid; PD, pyruvate decarboxylase; PK, pyruvate kinase; PPP, pentose phosphate pathway; SD, succinic dehydrogenase; TPP, thiamine pyrophosphate. (From Biale, J. and R. E. Young (1981) Respiration and ripening of fruits—retrospect and prospect, in *Recent Advances in Biochemistry of Fruits and Vegetables* (J. Friend and M. J. C. Rhodes, eds.), Academic Press, New York, p. 89.)

17.3.2.4 Lipid Metabolism

Although lipid components of most fruits and vegetables are normally present at relatively low levels, their metabolism is important during postharvest storage of plant tissues, especially when handling and processing conditions are not ideal. Changes in membrane lipids are also important in ontogenic events, such as stress responses, ripening, and senescence [35]. The aging process in plant tissues is associated with a decline in polyunsaturated fatty acids, and such changes apparently accompany autolysis of membranes and loss of cell integrity [38]. Phosphatidyl-linoleoyl (-linolenoyl) cascades

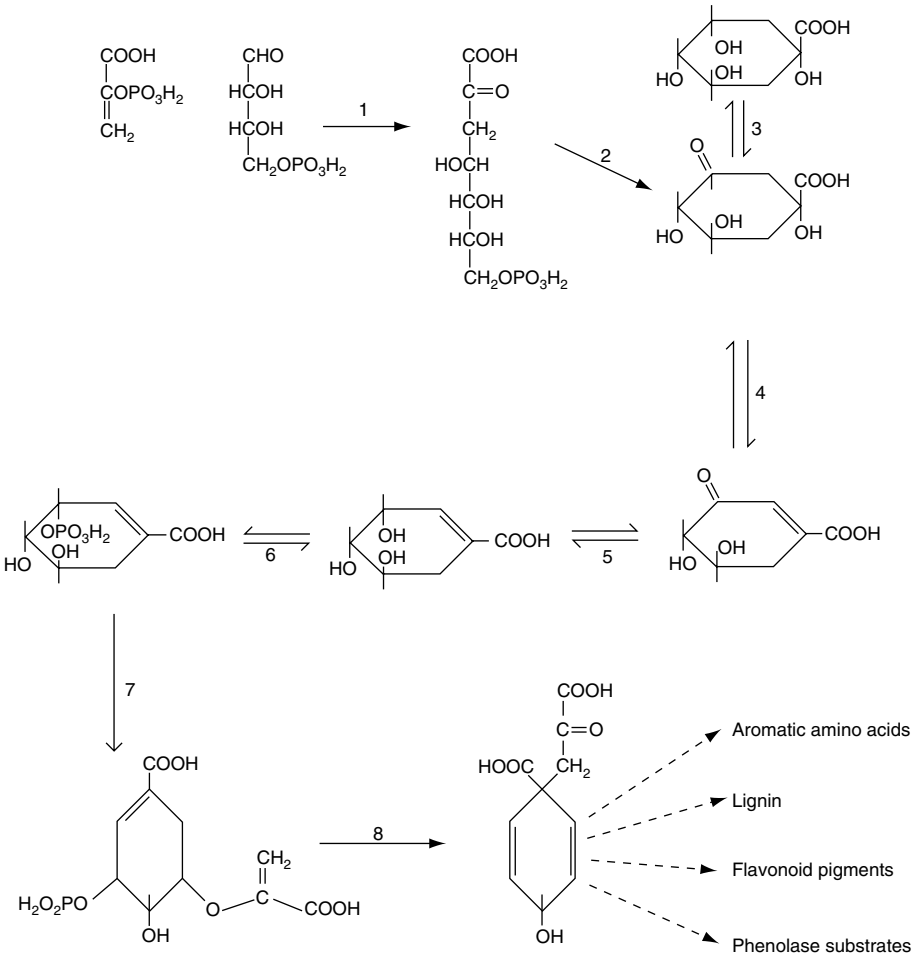


FIGURE 17.25 Biosynthesis of some important cell constituents via the shikimic acid pathway. (1) Phosphoenolpyruvate + D-erythrose 4-phosphate to 3-deoxy-D-arabinoheptulosonic acid 7-phosphate, (2) to 5-dehydroquinic acid, (3) to quinic acid, (4) to 5-dehydroshikimic acid, (5) to shikimic acid, (6) to 5-phosphoshikimic acid, (7) to 3-(enolpyruvate ether) of phosphoshikimic acid, and (8) to prephenic acid. The role of this pathway in the formation of phenolic compounds is detailed in Figure 17.21.

in plants also lead to the formation of a prostaglandin-like substance, jasmonic acid. Free radicals resulting from lipid oxidation act at sensitive subcellular sites and appear to play an essential role in normal plant development [74]. Free radicals are kept in check by scavengers such as superoxide dismutase, vitamins C and E, cytokinins, catalase, and glutathione peroxidase. Oxidative enzymes, such as lipoxygenase, hydroperoxide lyases, and hydrolytic enzymes, such as lipase and phospholipase, have also been shown to be important in postharvest metabolism. In fruits and vegetables, hydroperoxide lyases degrade specific hydroperoxides to form flavorful C_6 and C_9 volatile aldehydes and other products. Examples of flavor compounds formed by lipoxygenase cascades in injured and senescing plants include *t*,6-*cis*-nonadienal in cucumber, 9-hydroxy-*t*-10,*cis*-12-octadecadienoic acid in oats, and *cis*-3-hexenal and other compounds in tomatoes. Processes such as freezing and dehydration can activate lipoxygenase in unheated plant tissues and thus lead to off-flavor development. Hydroperoxides formed by lipoxygenase can also react with other constituents, such as proteins, and cause bleaching of carotenoids and chlorophylls.

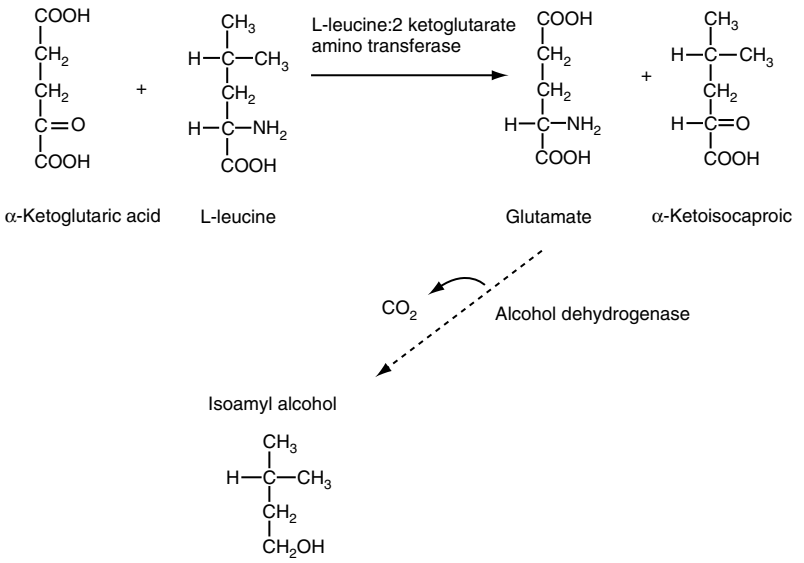


FIGURE 17.26 Biosynthesis of a tomato flavor compound from an organic acid and an amino acid.

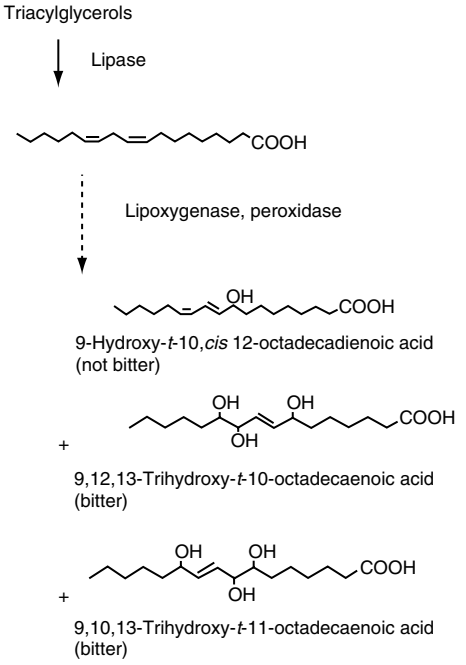


FIGURE 17.27 Formation of bitter trihydroxy fatty acids from triacylglycerols in processed oats.

The storage lipids (e.g., in palm, olive, and avocado) have compositions markedly different from those that are part of the functional cell framework. Enzymic hydrolysis of triacylglycerols in postharvest oilseeds has an important bearing on their quality. In comparison with other cereals, oats contain a high level of lipase, which together with lipoxygenase gives rise to bitter-tasting compounds (Figure 17.27).

17.3.2.5 Pigment Metabolism

Extensive biosynthesis of carotenoids and related terpenoids occurs in many edible plant tissues. Biosynthesis of these compounds occurs through the universal system for isoprene compounds and it involves the formation of mevalonic acid from acetyl-CoA. Most carotenoids are made up of eight isoprene units (C_{40}). Both biosynthesis and catabolism occur in detached plant parts and these reactions may be influenced by storage conditions such as oxygen concentration, light, and temperature. Carotenoid metabolism is affected by hormones, such as ethylene and abscisic acid. Some fruits and vegetables, such as mandarin orange, accumulate significant amounts of apocarotenoids (C_{30}). The most common catalytic agents for carotenoid destruction appear to be the lipoxygenases (indirectly via lipid oxidation) and the peroxidases (probably through secondary reactions).

The pathway for synthesis of the basic C_6:C_3 structure that comprises the A phenyl ring of the flavonoids is shown in Figure 17.25. Relatively little is known about reactions leading to the formation of specific flavonoid pigments. Synthesis of anthocyanins, colorants related to flavonoids, occurs in postharvest plant organs and is stimulated by light and influenced by temperature. The purple anthocyanin pigments of red cabbage are synthesized and accumulate in cabbage stored below 10°C. Preharvest treatments, such as application of *N*-dimethylaminosuccinamic acid, can induce early formation of anthocyanins in certain crops.

Catabolism of anthocyanin pigments *in situ* is not very well understood. In processed foods (e.g., during storage of frozen tissue that has not been blanched) anthocyanins can be co-oxidized by degradation products resulting from polyphenol oxidase or peroxidase-catalyzed oxidation of phenolic compounds. Decolorization of anthocyanins normally occurs following deglycosylation, and this is catalyzed by endogenous or fungal glycosidases called anthocyanases.

One of the more obvious changes that occur in senescing plant tissues containing chlorophyll is the loss of a characteristic green color. Peel and sometimes pulp degreening is also associated with ripening of most fruit, and "yellowing" is a characteristic of senescing stem and leaf tissues consumed as vegetables (Figure 17.9). Chlorophyll degradation *in situ* is accompanied by synthesis of other pigments in several kinds of detached plant tissues. Chlorophyll catabolism is markedly influenced by environmental parameters, such as light, temperature, and humidity, and the effects of these factors are specific for the tissue. For example, light accelerates degradation of chlorophyll in ripening tomatoes and promotes formation of this pigment in potato tubers.

Chlorophyll degradation in living plant tissues often can be promoted by application of parts per million levels of the hormone ethylene. It is common commercial practice to utilize this principle for degreening citrus fruit and ripening other commodities such as banana and avocado. Chlorophyllase emergence in plant tissues is associated with the buildup of endogenous ethylene. However, the relationship between chlorophyllase action and degreening is not clear. Chlorophyllase is a hydrolytic enzyme that converts chlorophylls *a* and *b* to their respective chlorophyllides. Present evidence suggests that chlorophyllase does not catalyze the initial step in chlorophyll degradation in senescing plant tissues. The initial steps in this process appear to require molecular oxygen, and removal of the phytol side chain occurs in later steps. Lipoxygenase and peroxidase are known to indirectly contribute to the loss of chlorophyll in senescing plants as well as in frozen vegetables. Chlorophyll conversion to pheophytin is acid catalyzed. This reaction is of primary importance in heat-processed foods, but it can also occur in living vegetables when they are stored in a carbon dioxide-enriched atmosphere.

Although enzymatic browning is a desired reaction in processes such as tea fermentation, drying of dates, and cocoa fermentation, it is a cause of discoloration when other tissues are subject to cutting, bruising, disease, freezing, or other causes of cell disruption. Substrates for this reaction include simple phenols (e.g., catechol and gallic acid), cinnamic acid derivatives (e.g., chlorogenic acid and dopamine), and flavonoids (e.g., catechin and epicatechin). These reactions are discussed in detail elsewhere (Chapter 6). The propensity of a given tissue to browning varies considerably from one cultivar of a crop to another. These differences appear to relate to variations in enzyme

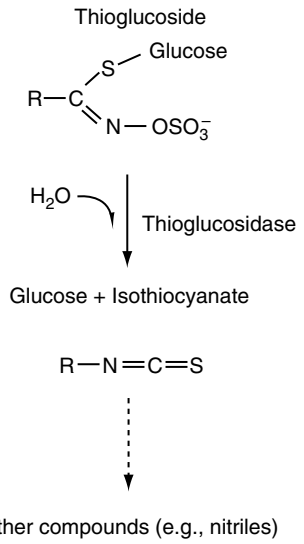


FIGURE 17.28 Biosynthesis of pungent mustard oils, characteristic of the *Cruciferae* family (Table 17.2).

content and sometimes to the kinds and amounts of phenolic substrates present. Methods used by the food processor to minimize this reaction include exclusion of oxygen, application of acidulants, heat inactivation (blanching), and use of inhibitors like sulfites.

17.3.2.6 Aroma Compounds: Biogenesis and Degradation

The characteristic flavor of fruits and vegetables is determined by a complex spectrum of organic compounds that form during maturation, senescence, and also sometimes during processing or wound injury. The total amount of carbon involved is much less than 1% of that evolved as CO_2. Aroma compounds in fruits and vegetables are generally a complex mixture of esters, aldehydes, ketones, terpenes, and others (Chapter 10). For example, more than 300 volatile compounds contribute to strawberry flavor. Typically, only a few key volatiles are important for the particular aroma of a given commodity. In some fruits and vegetables a single compound or class of compounds has the characteristic aroma, such as 2,6-nonadienal in cucumber, amyl acetate in banana, sulfides in onion, and phthalides in celery. Biosynthesis of fruit and vegetable flavors involves many different reactions that can include the metabolism or aromatic and *S*-containing amino acids (e.g., Figure 17.26), carbohydrates and their derivatives (Figure 17.28), and unsaturated fatty acids (Figure 17.27).

In certain tissues, such as *Allium* species and *Cruciferae*, flavor precursors are enzymically converted to flavor compounds when the cells are disrupted by chewing or other means of mechanical injury. A patented process exists whereby flavor-potentiating enzymes are added to processed foods, just prior to consumption, to regenerate the fresh flavor that was lost during heating or dehydration. This method takes advantage of the fact that precursor molecules may be stable to processing, whereas flavor compounds and enzymes are often labile. Lipoxygenase cascades also give rise to green, grassy, melon-like, and other aromas. The spectrum of compounds formed varies with different crops and depends on type of fatty acid(s) and on regiospecificity of lipoxygenases, hydroperoxide lyases, and/or peroxidases (Section 17.3.2.4; Figure 17.27). The presence of flavor precursors and their conversion to flavor compounds also has an important bearing on characteristic flavors that may develop as a consequence of different cooking and food preparation techniques (see also Chapter 10).

17.4 TECHNIQUES TO PRESERVE QUALITY AND EXTEND SHELF LIFE OF PLANT TISSUES

17.4.1 Maturity and Quality Standards

A high-quality horticultural product is one that possesses excellent or superior characteristics for its type. The term "quality" can be applied to various aspects of a commodity such as its visual appeal, flavor, nutritional content, texture, taste, freedom from chemical or biological contaminants, and so forth. The importance of each of these factors in the overall quality of a specific commodity varies with the commodity of interest. For example, fresh fruits and vegetables may rely heavily on visual and flavor quality, while ornamental crops primarily rely on visual quality, and processed products primarily rely on flavor. Often, visual quality ranks high in importance for most commodities, even though it may have little or nothing to do with the internal quality (taste, nutritional content, etc.) of the product. However, if anything makes the commodity unsafe (e.g., contamination with human pathogens), the product is immediately rendered unmarketable, regardless of its other quality characteristics.

Grade standards for perishable products provide criteria describing a commodity's level of quality to buyers and others in the marketing chain. Besides providing a common language between buyers and sellers, grade standards also assist in the settlement of claims and allow price reporting for comparing commodities of comparable quality. While U.S. grade standards are often optional, minimum maturity, or grade standards set by states, regions, or commodity marketing orders represent mandatory minimum quality standards that must be inspected by the USDA or affiliated agency. Individual buyers may require even higher standards that are negotiated between the seller and buyer.

Product maturity is a component of quality. Mature horticultural commodities are those that meet minimum acceptable quality standards following harvest, plus any required postharvest treatments (i.e., ripening). Optimal maturity of nonclimacteric fruits and vegetables generally coincides with optimal eating quality (quality does not improve postharvest). An exception might include sweetpotatoes, the eating quality of which improves after curing. The relationship between maturity and quality is more complicated for climacteric fruits, where the best eating quality occurs after ripening. However, the ultimate quality of climacteric fruit is still determined at harvest when the conduit for resources (e.g., sugars) entering the fruits is broken. Harvesting too early may result in insufficient fruits resources for ripening with acceptable quality. Most climacteric fruits attain their optimum eating quality when ripened on the plant (i.e., tree-ripe or vine ripe). The decision of when to harvest is often a compromise between maximum eating quality and the commodity's ability to survive the marketing chain—harvest too early, and the commodity never attains acceptable quality; harvest too late, and the commodity becomes too soft to survive the rigors of postharvest handling and transportation.

There are two types of maturity to consider when harvesting a commodity. Physiological maturity is the stage of development when a plant or plant part will continue normal, orderly development even if detached. For example, mature-green tomatoes are physiologically mature and can complete ripening even if detached from the plant. Horticultural maturity (also called commercial maturity) is the stage of development when a plant or plant part is at the stage desired by the consumer (e.g., physiologically mature red tomatoes, physiologically immature florets of broccoli). Many horticulturally mature commodities (e.g., cucumbers and broccoli) are not physiologically mature.

Determining the proper time for harvest is the first step in successfully delivering high-quality produce to the consumer. The proper maturity at harvest is very important because it is at this developmental stage that edible plant organs have their optimum potential for quality—that potential can only decline following harvest. The proper harvest maturity varies with different commodities and their intended uses. For example, many pome and stone fruits reach their best eating quality when fully ripened on the tree while pears (also a pome fruit) tend to become mealy in texture if ripened on the tree. In addition, mature-green tomatoes are firmer and can better survive the rigors of shipping while fully red-ripe tomatoes are softer and better suited for processing.

In any commercial operation, the ability to predict when a commodity will reach optimum harvest maturity is vitally important so that the appropriate resources and preparations can be made prior to harvest. For example, harvesters and their equipment must be available, packinghouse equipment must be ready to handle the fruit, storage or ripening areas must be prepared and available, and buyers must be identified to purchase the product. Postharvest delays due to any of these result in reduced quality and storage life. Besides predicting optimum harvest maturity, these indexes should also be relatively simple to measure, be objective, and not be affected by grower, growing location, or production year. A variety of maturity indexes have been devised for different commodities based on factors such as size, shape, texture, solidity, external and internal color, composition (sugars, acids, total solids, etc.), specific gravity, abscission layer development, elapsed days from full bloom, and heat units during development. Some of these measurements are destructive (e.g., tissue must be macerated for most compositional analyses), while others are nondestructive (e.g., for specific gravity, external color, etc.). Recent work has focused on nondestructive technologies to quickly and directly measure the quality of each fruit or vegetable being packed. For example, near infrared sensors on packingline equipment now allows product grading based on internal sugar concentration so that buyers can purchase commodities with guaranteed minimum sugar content. This ability to grade individual fruit based on internal quality has allowed the sale of some fruit that normally would not be acceptable to various fresh markets because of external blemishes.

17.4.2 Temperature Management

Temperature is the most important environmental factor determining the postharvest life of a commodity. Because the rate of respiration decreases about two- to threefold for every 10°C decrease in temperature, low temperatures have a profound effect on decreasing respiration and thus increasing the storage life of various commodities. In addition, reduced temperatures can also dramatically reduce pathogen growth and affect other internal and external factors (such as reducing water loss and influencing compositional changes). Therefore, it is desirable to maintain storage and holding temperatures as low as possible without causing freezing injury, CI, or undesirable compositional changes (e.g., starch to sugar conversion in potatoes).

After harvest, it is important to cool perishable commodities as soon as possible. Warm crops from the field have high rates of respiration and, if not cooled, will continue rapid physiological aging leading to accelerated senescence of the tissue. Rapid cooling removes field heat (also called sensible heat) and reduces heat released by respiration (vital heat). Even a few hours delay in cooling, can result in marked reductions in storage life.

17.4.3 Cooling Methods

Commercially, there are several types of cooling methods that are utilized, each with their own variations. The particular choice of which method to use depends on many factors such as speed of cooling (Figure 17.29), commodity requirements, cost, portability, and so forth.

17.4.3.1 Air Cooling

In this method, air is used as the cooling medium. These cooling systems are relatively inexpensive because air is free and easy to move around. However, because of the low thermal capacity and conductivity of air, cooling can take a long time. Two types of air cooling are room cooling and forced air cooling.

17.4.3.1.1 Room Cooling

Room cooling involves simply placing the product into a refrigerated room and allowing the product to cool by the passive movement of cold air around the product (Figure 17.30). This method requires

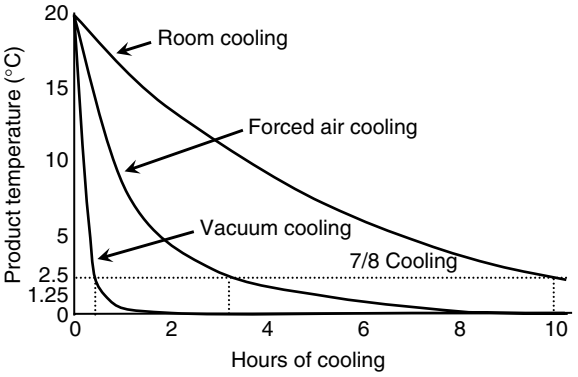


FIGURE 17.29 Generalized cooling curves for room, forced-air, and vacuum-cooling systems.

FIGURE 17.30 Room cooling. Refrigerated air is circulated throughout the room using the evaporator fans mounted near the ceiling in the back of the room. Cooling is only through passive convection. In this case, the small area for carton ventilation will result in very slow cooling times.

less refrigeration capacity than other cooling methods because heat removal is spread over longer periods (i.e., overnight). The product can also be stored where it is cooled. However, it is the slowest of all the cooling methods.

17.4.3.1.2 Forced Air Cooling

Forced air cooling involves actively moving cold air over the product by using a pressure gradient to force air through the product containers (Figure 17.31). This method is the most widely adapted cooling method for different commodities and is much faster than room cooling (taking typically only one-fourth to one-tenth as long). Product can be shipped faster, taking up less floor space, and so forth. However, it requires larger refrigeration capacity to handle peak heat removal.

FIGURE 17.31 Tunnel-type forced-air cooling. Two rows of product in ventilated containers are arranged so that an air-return pathway between the rows is left open to the refrigeration coils. A canvas is draped over the product to force air returning to the refrigeration coils to pass through the product. Note air returning under the pallets is blocked by excess canvas in front, and by blocking materials on the sides. Air returns to the room through the opening in the back wall, above the product.

Water loss can be a problem if product cooling takes too long (room cooling), or if rapid air movement over the commodity is continued after the product is cooled (forced air cooling).

17.4.3.2 Hydrocooling

Unlike air, water's high-thermal capacity makes it an efficient medium for cooling product. In addition, water loss from the commodity is prevented during cooling. However, products and their containers must tolerate free water and any chemicals (e.g., sanitizers) in the water. Hydrocoolers commonly cool by drenching product with cold water (Figure 17.32), or by immersing product in cold water. In some places, even cool well water can be used for initial product cooling. Immersion-type hydrocoolers generally do not work well for product that floats or that have large intercellular spaces that promote uptake of solution during cooling.

Because water is such a good vector for pathogens, maintenance of good water sanitation is required. Thus, recirculated water systems should be treated with chlorine or other sanitizer and the water monitored frequently and changed regularly to reduce pathogen levels in the water.

17.4.3.3 Ice Cooling

Ice should be one of the most efficient methods of cooling because of its high heat of fusion which absorbs 80 cal/g of heat as it melts. However, it is difficult to obtain complete contact between the ice and the product, which results in uneven cooling. Techniques that increase product contact with the ice (e.g., use of small pieces of ice, alternating layers of ice and product, liquid ice, etc.) speed cooling (Figure 17.33). Similar to hydrocooling, product and containers must tolerate free water, chemicals, and good water sanitation is required. Other problems include the need to ship added weight (ice), the mess (sanitation issue) of leaking of water as the ice melts from the containers, and the need for periodic renewal of ice in the containers.

17.4.3.4 Vacuum Cooling

Vacuum cooling (Figure 17.34) is unique in that it uses no recirculated cooling medium (e.g., air or water) to cool the product. Instead, product is placed in large, air-tight chambers, and a vacuum is

FIGURE 17.32 Conveyer hydrocooling using ice-cold water pumped into a pan over the product that is then showered down through the product.

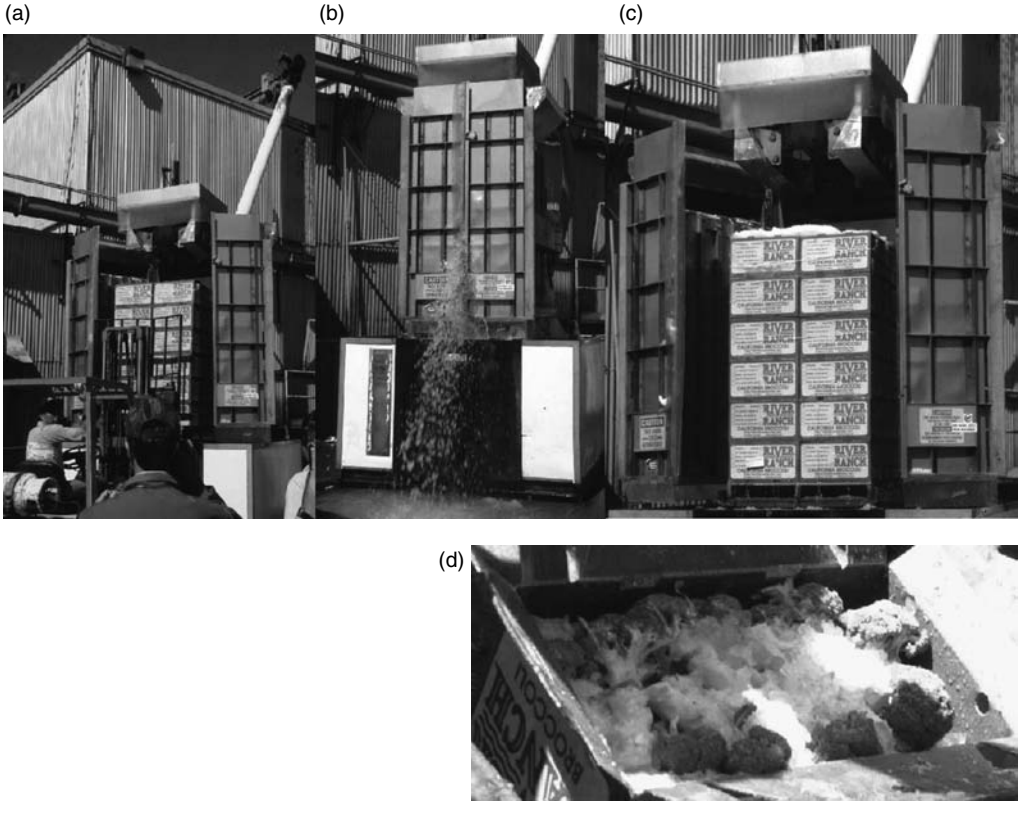


FIGURE 17.33 Liquid ice injecting. Pallets of product that are packed in the field arrive at the cooler and are loaded into the injector (a). A slurry of water and ice is passed through the pallet (b) from top to bottom leaving ice distributed throughout the cartons (c and d).

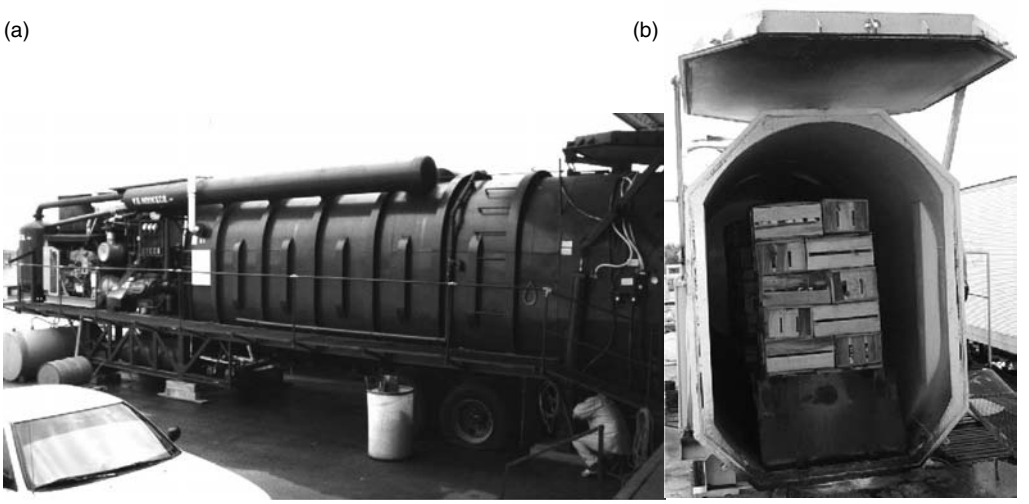


FIGURE 17.34 Mobile vacuum cooler used for leafy vegetables. Side (a) and end (b) views. Product is loaded into the chamber and a vacuum drawn until water evaporates at near 0°C, cooling throughout the product.

applied to the contents. At about 5 mm of mercury, water boils at about 0°C, and as it evaporates, water's latent heat of vaporization removes about 580 cal/g from the product. This is most beneficial in commodities with high surface-to-volume ratios, such as lettuce, where it is able to evenly cool the center of the product. Because water is lost during the process, water is sometimes sprayed on the commodity before cooling so that the water used for cooling comes primarily from the added water, and not the commodity itself.

17.4.4 Storage and Transportation; Modified and Controlled Atmospheres

A range of techniques is available to store perishables and, for many fruits and vegetables, the time spent in transit from field to consumer represents the majority of their postharvest life and is de facto storage. By far the majority of fruits and vegetables are stored in insulated rooms and transported in insulated trailers and marine containers provided with mechanical means of refrigeration, while grains are commonly held in ambient conditions. Best product condition is maintained under constant refrigerated temperatures. Typically, the temperature during handling of fruits and vegetables should vary no more than ±1°C from the desired temperature; even this may be excessive near the freezing point of the commodity. Large swings in temperature can result in freezing or CI, accelerated water loss, and reduced market life.

17.4.4.1 Humidification

For most perishable products a RH of 90–95% is recommended during storage. Very high RH (close to 100%) may cause cracking of some commodities, and surface condensation on the product may accelerate growth and spread of microorganisms.

17.4.4.2 Transportation

Road transport in refrigerated trucks accounts for well over 80% of the movement of perishable products in the United States. For international trade in perishable commodities, sea transportation, primarily in 55–65 m^3 refrigerated "containers," is the preferred method. Both trailer and container

refrigeration systems are designed for temperature maintenance only, and can thus be easily overwhelmed if the load is not adequately cooled prior to stowage. Airfreight is used for products with a very short storage life, such as strawberries and cut flowers. The advantage of air transport is speed, however, temperature management is rudimentary, and delays at both departure and destination terminals can be substantial. Lack of temperature control in loading areas can cause heating or freezing of the product.

17.4.4.3 Controlled and Modified Atmospheres

CA storage refers to storage systems in which the composition of the atmosphere external to the commodity has been altered with respect to the proportions of O_2 and/or CO_2, and in which the gas concentrations are continuously monitored and actively controlled, usually within $\pm 1\%$ of the desired value. The use of CA for maintenance of high-quality produce during storage and transportation grew out of research in England by Kidd and West following the food shortages during World War I, who discovered that certain varieties of apples remained in better condition in atmospheres that contained less oxygen and more carbon dioxide than air [32].

Modified atmosphere storage does not differ in principle from CA, except that control of gas concentration is less accurate. The atmosphere in MA systems may be purposely established, but is passively maintained by a balance between commodity respiration and restricted diffusion in a package, transport vehicle, or storage room. For example, CO_2 may be allowed to simply accumulate in railroad cars, either by normal respiratory activities of plant tissue or by sublimation of dry ice (solid CO_2) used for cooling.

The diffusion gradient for movement of gases into or out of a commodity depends on its surface to volume ratio, resistance to diffusion (a function of number and size of stomata, lenticels, breaks in the epidermis, and cuticle structure), rate of production or consumption of each gas by the commodity, and the difference in partial pressures of these gases inside and outside the tissue. Three levels of barriers to gas exchange can be envisioned in a fruit or vegetable storage [30] (Figure 17.35): (1) the structure of the commodity dermal system and added surface coatings, (2) packages with semipermeable properties, and (3) the gas tightness of storage rooms or transit vehicles. These barriers

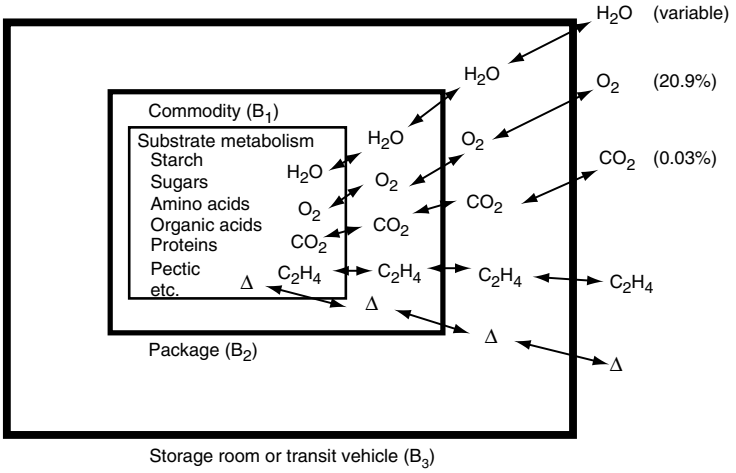


FIGURE 17.35 Model showing three levels of barriers to gas exchange in CA storage of a commodity. B_1 = commodities dermal system and added barriers (e.g., waxing, film wrap, edible coating); B_2 = packaging material or CA storage room; B_3 = storage room or external environment. (Adapted from Kader, A. A. and M. E. Saltveit (2003) Respiration and gas exchange of vegetables, in *Postharvest Physiology and Pathology of Vegetables*, 2nd edn. (J. A. Bartz and J. K. Brecht, eds.), Marcel Dekker, New York, pp. 7–29.)

determine the composition of the atmosphere in these chambers and ultimately the atmosphere within the plant tissue.

17.4.4.4 Benefits of MA and CA

The beneficial effects of CA and MA include retardation of senescence (including ripening) and associated biochemical and physiological changes (i.e., slowing down respiration, ethylene production, softening and compositional changes), inhibition of ethylene synthesis and reduction of product sensitivity to ethylene action, and reduction of decay caused by microorganisms. Although the efficacy of CA techniques for extending storage life has been demonstrated for many crops, commercial use has been somewhat limited, either for economic reasons or because rapid turnover of a commodity in the marketplace lessens the need for improved storage procedures. Conventional CA storage (large facilities) is used routinely for apples and pears and use of MA in retail packages has increased, especially for highly perishable fresh-cut products. The latter approach is achieved by product-generated respiratory gases and the use of packaging materials with desired gas permeability. Hypobaric storage has been employed successfully by commercial growers of cut flowers, but has not been employed commercially for the storage or transportation of fruits or vegetables primarily due to higher associated costs for hypobaric compared to conventional CA. Maintenance of temperature in MA packaged produce is critical because small changes in temperature can have large effects on respiration of the product and little effect on the permeability of the packaging.

17.4.4.5 CA-Related Disorders

Different fruits and vegetables have different, very specific, and often unpredictable tolerances to low concentrations of O_2 and high concentrations of CO_2. The degree of susceptibility to injury and the specific symptoms vary, not only among cultivars, but also among the same cultivars grown in different locations or different years in the same location. Differences in susceptibility to elevated CO_2 and/or reduced O_2 levels among commodities or among cultivars of a given commodity may be due to structural (anatomical) or metabolic differences. The maturity of the tissue at harvest and the temperature of the tissue during storage can also influence the response to CA conditions. With respect to maturity, "McIntosh" apples, when stored in an atmosphere containing 1% O_2, exhibit more internal browning as their maturation advances.

If the composition of the storage atmosphere is not properly controlled it can have disastrous results, since the produce may develop physiological injuries. Some crops, such as broccoli, strawberries, and sweet cherries, are not very susceptible to CO_2 injury and can be stored at CO_2 concentrations up to at least 20%, whereas other crops, such as lettuce, can show discoloration ("brown stain") when exposed to CO_2 concentrations of 2% or greater. Similarly, atmospheres containing large amounts of CO_2 and small amounts of O_2 interfere with wound healing of potato tubers. Thus, wounded potatoes decay very rapidly if exposed to CA immediately after harvest. Other symptoms of damage by extreme atmospheres in CA and MA include irregular ripening, development of off-flavors and off-odors (related to anaerobiosis/fermentation), increased susceptibility to decay, and stimulation of sprouting.

The mechanism by which physiological disorders are induced by extremes in atmospheric composition is not entirely clear. However, available evidence indicates that some of the same principles discussed in reference to CI also apply to CO_2 injury or damage caused by O_2 deprivation. Studies have shown that toxic levels of metabolites, such as succinic acid, ethanol, and acetaldehyde, accumulate in certain commodities prior to injury symptoms. It is, of course, very difficult to distinguish primary sites of injury from secondary consequences of the primary lesion. However, it is reasonable to believe that alterations in respiratory metabolism and the inability of the tissue to cope with the resulting imbalances are a primary event in such injuries.

Although the final electron acceptor for respiratory metabolism (cytochrome oxidase) has a relatively high affinity for O_2, it is important to recognize that the gaseous concentrations *in situ* are not the same as those existing in the storage atmosphere. Thus, the diffusivity and solubility of a critical gas in the tissue may be determining factors.

17.5 COMMODITY DESCRIPTIONS

17.5.1 Cereals, Pulses, and Oilseeds

Unlike fruits and vegetables, cereals, pulses, and oilseed are relatively stable during storage, provided the moisture level is below a critical value—typically about 14%, but this varies somewhat with the crop. Above this moisture content, respiration, mold growth, and sprouting become problems. The higher the moisture content above the critical value, the greater are the problems. Changes in quality of these crops during storage under proper conditions are quite subtle and sometimes may not be obvious. The hard-to-cook phenomenon in dried beans, which results in beans that need substantially more cooking time than normal beans, is the result of membrane changes that occur during storage at higher than optimum humidity [55].

Milling, crushing, or extraction greatly reduces storage stability of cereals, pulses, and oilseeds. Destruction of cellular compartmentation results in mixing of enzymes and substrates and exposure of cellular contents to oxygen and microorganisms. For products with a moisture content yielding optimum overall stability, the rate of lipid oxidation is the limiting factor for storage. The quality of wheat flour for baking increases after milling due to oxidative changes in the protein, but once optimum quality is achieved further storage causes a slow reduction in quality [49].

17.5.2 Vegetative, Floral, and Storage Organs

Not all "vegetables" are composed of vegetative parts of plants, but those types do include leafy crops like lettuce, cabbage, Brussels sprouts, celery, rhubarb, spinach, chard, kale, endive, and escarole; and stem vegetables like asparagus and kohlrabi. Many vegetables are underground storage organs, including roots like carrot, radish, turnip, table beet, and sweetpotato, tubers like potato, and bulbs like onion and garlic. Potato is the most widely consumed vegetable worldwide. A few vegetables are actually immature floral organs such as artichoke, broccoli, and cauliflower. The leafy and floral vegetables are much more perishable than the root vegetables because they are typically still developing when harvested and have much higher metabolic rates. Major causes of deterioration include water loss (wilting), loss of chlorophyll (yellowing), mechanical injury, and physiological disorders. Morphological changes can also be important in some commodities (e.g., growth and toughening of asparagus, celery, etc.). The principle causes of postharvest losses of the "root" vegetables are mechanical injuries, improper curing, sprouting and rooting, water loss, CI, and decay. See Bartz and Brecht (2003) for a thorough review of the physiology and handling requirements of fresh vegetables.

These crops exhibit nonclimacteric respiratory behavior and produce very little ethylene. Most leafy and immature floral vegetables are high in respiration rate while the root crops are lower. The root vegetables are "storage organs" for the plant and serve as propagules. Hence they resist deterioration and are well adapted to long-term holding. The leafy and immature floral vegetables have minimal or no storage reserves and lose these rapidly due to their high respiration rates. The leafy and immature floral vegetables can be divided according to rate of respiration (heat production) as very high (>40 mg CO_2/kg-h at 5°C) including asparagus, spinach, turnip greens, and broccoli; high (20–40 mg CO_2/kg-h at 5°C) including artichoke, Brussels sprouts, and leaf lettuce; and moderate (10–20 mg CO_2/kg-h at 5°C) including cabbage, cauliflower, celery, kohlrabi, and head lettuce. Based on respiration rates, the root vegetables can be divided into two groups: very low rates (<8 mg/kg-h at 0°C) including beet, parsnip, potato, onion, celeriac, and turnip; and low rates

(8–12 mg/kg-h at 0°C) including carrot, radish, and horseradish. Root crops marketed with tops have a higher respiration rate than that of the roots alone (e.g., carrots with tops [35 mg CO_2/kg-h at 0°C] vs. carrot roots alone [13 mg CO_2/kg-h]). Potatoes harvested immature (new potatoes) respire twice as fast as those harvested mature; and cured potatoes and sweetpotatoes have lower respiration rates than non-cured.

17.5.3 Immature and Mature Fruit Vegetables

These crops are botanically fruit, but are considered vegetables in common usage because of how they are grown and consumed. Vegetables that are immature fruits include fleshy fruits such as cucumber, soft-rind (summer) squash, eggplant, and green pepper, and also nonfleshy fruits such as snap beans, lima beans, southern peas (cowpeas), peas, broad beans, sweetcorn, and okra. Mature fruit vegetables include fleshy fruits such as tomato, red pepper, hard-rind (winter) squash, pumpkins, muskmelons, watermelons, and nonfleshy dry peas and beans. Tomato is the second most widely consumed vegetable worldwide after potato; partly due to this high consumption, tomato ranks #1 in relative contribution to nutrition. Cantaloupes are high in Vitamin A contribution to human diet and peppers are highest in Vitamin C content among this group; legumes are major contributors of protein, niacin, thiamine, and some minerals. The fruit vegetables are not adapted to long-term storage except for hard-rind (winter) squash, pumpkins, and dry legumes.

Most of these fruit vegetables exhibit nonclimacteric respiratory behavior; tomatoes and cantaloupes are the only climacteric fruits in the group and honeydews do not exhibit a consistent climacteric. Respiration rates vary from very high (>100 mg CO_2/kg-h at 10°C) for sweetcorn and peas to low (<10 mg CO_2/kg-h at 10°C) for honeydew and watermelon. Ethylene production rates are very low (<0.1 μL/kg-h) in immature fruit vegetables and nonclimacteric mature fruit vegetables. Ethylene production rates may reach high levels (>20 μL/kg-h) in ripening tomatoes and cantaloupes. For fruit vegetables harvested mature, satisfactory ripening occurs only within the limits of about 12–25°C and optimum ripening temperatures are 20–22°C.

17.5.4 Small Fruits, Pome, and Stone Fruits

Small fruits include all types of grapes, kiwifruit, and berries. Most fresh market grapes are now seedless (e.g., "Thompson Seedless," "Flame Seedless," etc.), but some seeded cultivars are still important. The kiwifruit are unique in this group of fruits in that they accumulate a high starch content that is converted to soluble solids as the fruit ripen. The "berries" include blackberry, blueberry, cranberry, raspberry, strawberry, and others.

Except for kiwifruit and blueberries, small fruits are nonclimacteric and are harvested close to their optimum eating quality since they do not undergo postharvest ripening. Grapes, cranberries, and kiwifruit have relatively low rates of respiration (5–10 mg CO_2/kg-h at 5°C) compared to blueberries and gooseberries (10–20 mg CO_2/kg-h), and the high-respiring blackberries, raspberries, and strawberries (20–40 mg CO_2/kg-h). In grape bunches, respiration of the stems may be 15 times greater than the berries. Other than kiwifruit with high ethylene production when ripe (10–100 μL C_2H_4/kg-h at 20°C) and blueberries with moderate ethylene production (1–10 μL C_2H_4/kg-h), most small fruits produce little ethylene (<1 μL C_2H_4/kg-h).

After harvest, kiwifruit soluble solids content (SSC) may more than double as starch is converted to simple sugars. In addition, the fruits experience dramatic postharvest softening in response to even trace amounts of ethylene (5–10 ppb) in the atmosphere. Interestingly, however, the climacteric does not occur until well after the fruits are soft enough to eat [56].

Grapes, kiwifruit, and berries are all very susceptible to water loss. Even as little as 1% water loss in strawberries can result in noticeable losses in surface sheen. Water loss leads to grape stem browning, berry shatter, and berry shrivel. For all, rapid cooling is an important tool for minimizing water loss.

Pome fruits include apples, pears (European and Asian), and Quince. Both apples and pears are climacteric fruits, but some Asian pears are nonclimacteric. Apples generally exhibit low rates of

respiration (5–10 mg CO_2/kg-h at 5°C), whereas pear respiration is moderate (10–20 mg CO_2/kg-h). Both apples and pears have high ethylene production rates (10–100 μL C_2H_4/kg-h at 20°C) and exposure to ethylene hastens the climacteric and ripening. Reducing respiration, through exposure to low temperatures and CA or MA storage, or delaying the climacteric by reducing ethylene exposure or sensitivity has greatly extended the storage life of apples.

Stone fruits include the peaches, nectarines, plums, apricots, and sweet cherries. Peaches, nectarines, and plums are available over a relatively long period (~5 months) because of the large number of cultivars (often >100) that ripen over an extended period. There are fewer cultivars of apricot and cherry resulting in a shorter period of availability.

Peaches, nectarines, plums, and apricots are climacteric fruits with high rates of ethylene production (10–100 μL C_2H_4/kg-h at 20°C), while sweet cherries are nonclimacteric with low rates of ethylene production (<0.1 μL C_2H_4/kg-h). All have moderate rates of respiration (10–20 mg CO_2/kg-h at 5°C).

17.5.5 Subtropical Fruits

Subtropical fruits are a diverse group and include avocado, cherimoya, citrus fruit (orange, grapefruit, lemon, lime, tangerine, pummelo, and kumquat), date, fig, jujube (Chinese date), longan, loquat, lychee, olive, persimmon, and pomegranate. They represent diverse morphology, physiology, and compositional characteristics that result in diverse optimum postharvest handling requirements.

Avocado, cherimoya, fig, and persimmon are climacteric, whereas date, jujube (Chinese date), longan, loquat, lychee, olive, pomegranate, and all citrus are nonclimacteric. Respiration and ethylene production rates vary considerably, ranging from very low respiration in dates, to high respiration in avocado, and very low ethylene production in pomegranate and nonclimacteric fruits, to high ethylene production in avocado. Perishability also varies considerable from highly perishable fruits such as fig with a shelf life of <2 weeks, to less perishable fruits such as date that can be stored for up to a year.

Though citrus is nonclimacteric and produces very little ethylene, ethylene is often used to stimulate the loss of green chlorophyll in the peel (degreening) to allow the orange and yellow carotenoids to predominate. Degreening is most common early in the season when natural degreening has been delayed because of warm night temperatures. Degreening usually takes 1–3 days to complete and does not affect internal quality (e.g., SSC, titratable acidity, etc.). However, ethylene does stimulate decay and fruit respiration and so should be kept to a minimum. Specific conditions for degreening differ by growing region (Table 17.18).

17.5.6 Tropical Fruits

Tropical fruits include banana, breadfruit, carambola, durian, guava, jackfruit, mango, mangosteen, papaya, passion fruit, pineapple, prickly pear, rambutan, sapodilla, soursop, and sweetsop.

Banana, breadfruit, carambola, durian, guava, jackfruit, mango, mangosteen, papaya, passion fruit, rambutan, sapodilla, sapote, soursop, and sweetsop are climacteric, whereas pineapple and

TABLE 17.18
Recommended Degreening Conditions for Florida and California Citrus

Factor	Florida	California
Temperature (°C)	28–29	20–25
Ethylene (ppm)	5	5–10
Relative humidity (%)	90–96	90
Ventilation (air change per hour)	1	1–2
Air circulation	100 ft^3/min per 900-lb bin	1 room volume per min

prickly pear are nonclimacteric. Respiration and ethylene production rates vary considerably, ranging from low respiration in pineapple and papaya, to very high respiration in passion fruit, and low ethylene production in prickly pear and pineapple, to very high ethylene production in passion fruit and sapote. Perishability ranges from 1 to 8 weeks.

17.5.7 Fresh-Cut Fruits and Vegetables

Fresh-cut fruits or vegetables, which have been trimmed, peeled, and/or cut into a ready-to-eat fresh product, are an increasingly important part of the plant food industry. Examples include salad mixes; shredded, chopped, halved, and cored lettuce; broccoli and cauliflower florets and slaws; cabbage shreds and fresh coleslaw; baby carrots and shredded carrots; celery sticks; squash and zucchini (slices); fruit salads; washed and de-stemmed grapes; cantaloupe, honeydew, and watermelon halves and cubes; pineapple slices and cubes; and apple, nectarine/peach, mango, and papaya slices.

The injuries to which fresh-cut produce items are subjected in their preparation, trigger shifts in the metabolism of the injured tissues that result in accelerated respiration, wound ethylene production, senescence, ripening, and deterioration. Removal of the epidermal layer also exposes interior tissues to oxidation and browning, increases water loss, and provides easier access for infestation by microorganisms. This means that fresh-cut products are much more perishable than their intact counterparts. The visual symptoms of deterioration of fresh-cut produce include flaccidity from loss of water, changes in color (especially increased oxidative browning at the cut surfaces), and microbial contamination. Fresh-cut fruit products must be fully ripe and ready to eat when the consumer opens the package, which adds to the perishability of such products.

Microbial contamination and growth on or in fresh-cut vegetables and fruits is a major concern since certain human pathogens can also grow or survive in fresh-cut vegetables and fruits. Rigorous sanitation of preparation areas reduces the level of microbial contamination, while chemical treatments and low temperatures restrict microbial growth during storage and marketing. Pathological deterioration of fresh-cut products is of less practical concern because fresh-cut shelf life is almost always limited by physiological rather than microbiological breakdown.

Temperature management is extremely critical for fresh-cut fruits and vegetables. Fruits and vegetables to be used for fresh-cut products are precooled and the processing facility is maintained at 2–7°C. Newly prepared fresh-cut products are immediately washed and rinsed in chilled (0°C) water, and then they are quickly packaged before being held at 1–5°C in order to minimize the negative consequences of processing. Edible coatings are sometimes applied to fresh-cut products as a barrier to O_2 movement but can also serve as a carrier for antimicrobials and firming agents, or chemicals that improve oxidative stability or inhibit oxidase enzymes. Fresh-cut vegetables and fruits are almost universally handled in MA packaging (MAP), in which film permeability, film area, and product respiration rate interact to create a beneficial atmosphere. Using MAP helps maintain fresh-cut product quality and extend shelf life by inhibiting metabolic activity, growth of microbes, browning reactions, and especially by inhibiting ethylene biosynthesis and action.

17.6 EFFECTS OF PROCESSING ON FRUITS AND VEGETABLES

The basic purpose of food processing is to curtail the activity of microorganisms and retard chemical changes that would otherwise adversely affect the edible quality of food, and to do so with minimal damage to quality attributes. Food processing techniques differ in principle from the preservation techniques described in the previous section in that viability of the living tissue cells is lost during processing. An important advantage of processing is that it enables food to be stored in edible condition for longer periods than would generally be possible by other means. However, because of the severity of these processes, the product usually undergoes rather extensive changes, and as a result the quality characteristics of a given commodity are often changed. Such alterations may

be desirable (e.g., inactivation of antinutritional factors by heat, softening of hard or tough tissues, creation of flavors) or undesirable (e.g., loss of vitamins by heat, loss of color, texture changes, and production of off-flavors) and may be unacceptable to consumers depending on local customs and eating preferences.

17.6.1 Chemical Changes

The rate of a chemical reaction in a foodstuff is a function of many factors, mainly reactant concentration, availability and mobility; temperature; pH; oxidation–reduction potential; and inhibitors and catalysts. Therefore, the effects of processing on chemical changes in fruits and vegetables vary widely with the nature of the process and composition of plant tissue. Even within the same species, substantial differences may occur among different cultivars and for a single cultivar that has been grown or stored under different conditions. The reactions that occur in plant tissues are not unique to plants except for those that occur while the tissue is still "alive" and those that involve components that are specific to plants. Thus, chemical alterations in starch that occur during canning of whole potatoes are not fundamentally different from those occurring when potato starch is canned as a part of a soup. However, there are important physical differences that are related to whether or not the starch granule is a part of a complex cellular organization or simply dispersed in water, and these physical differences may affect the rate and extent of chemical reactions. Some examples of reactions that occur during processing and storage of fruits and vegetables are shown in Table 17.19. Because of the complexity of fruit and vegetable tissues it is often difficult to predict the consequences of processing and storage conditions on chemical reactions that influence quality. The details of many of these chemical reactions can be found in the chapters dealing with the individual components.

17.6.2 Enzyme-Catalyzed Reactions

The effects of cellular disruption on chemical reactions in living plant tissues have been mentioned several times in this chapter. Even when foods are to be thermally sterilized, cellular disruption during peeling, size reduction, and so forth, before the enzymes are inactivated contributes significantly to the chemical changes that occur. Many processing sequences are designed to minimize these changes by reducing the time between disruption and enzyme inactivation. This is one of the functions of blanching prior to thermal processing or freezing. The "hot break" process for tomatoes, which inactivates PG before significant losses of viscosity occur, is a good example of this type of strategy. However, in the processing of orange juice, some of the PGs are quite heat stable, and substantial changes in flavor occur when all of the PG is inactivated by heat. Failure to inactivate PG and PME in orange juice results in an unstable "cloud" and a poor appearance.

17.6.3 Cell Disruption

Cellular disruption also contributes to nonenzymatic changes. Disruption of the central vacuole releases acid, which changes the pH of the tissue and alters the rates of numerous pH-dependent reactions. Color changes are especially noticeable examples of this. Disruption may also increase the rate of nonenzymatic oxidation due to the increased concentration of oxygen. When freezing plant tissues, it is generally agreed that ice formation directly causes physical disruption of the relatively rigid plant cell structure and indirectly causes damage due to the concentration of solute molecules in the unfrozen phase. Tissues vary widely in their susceptibilities to these kinds of damage. Differences in freezing damage to a leaf or tomato as compared to that in a potato or wheat kernel illustrate this point. Due to the effects of temperature, rates of reaction following cellular disruption during freezing are quite different from those that occur following disruption at higher temperatures where enzymatic and chemical reactions are rapid.

TABLE 17.19
Examples of Nonenzymic Reactions That Can Affect the Quality of Processed Fruits and Vegetables

Reactant(s)	Product(s)/Result	Importance
Chlorophyll	Pheophytin	Conditions of low pH and high temperature favor this reaction, which results in olive-brown discoloration of canned green vegetables
Glutamine	Pyrrolidone carboxylic acid (PCA)	PCA formed during thermal processing of canned vegetables is believed to contribute to acid-catalyzed pheophytin formation and off-flavor
All-*trans*-carotenoids	*Cis*-, *trans*-carotenoids	Heat, light, or dilute acid conditions cause isomerization of all-*trans*-carotenoids to various *cis* isomers. The reaction results in loss of vitamin A activity and changes in color
Thiamin	Pyrimidine and thiazole	Heating at pH < 6 results in cleavage of the methylene bridge of vitamin B_1 to form the indicated products
Ascorbic acid	Dehydroascorbic acid, diketogulonic acid, etc.	Oxidation in the presence of molecular O_2 results in loss of vitamin C activity and may be coupled to other redox reactions, such as disulfide bond formation during gluten development, or conversion of ethanol to acetaldehyde in wine aging
Ascorbic acid	3-Deoxypentosulose, 2-furaldehyde	High-acid foods like lemon juice undergo nonoxidative degradation of ascorbic acid. The reaction appears to contribute to "ascorbate browning" of these products
Glycosides	Hydrolysis products	Mild acid conditions and heat cause hydrolysis of glycosidic linkages. Phenolic glycosides are also hydrolyzed under mild alkaline conditions. Such reactions can influence the texture and flavor of fruits and vegetables
Reducing sugar, amino acid	Maillard browning	Concentration of reactants, high temperature, and alkaline pH can favor this important reaction that can affect color, flavor, and safety, and adversely affect nutrition of processed fruits and vegetables
Organic acid, Ca^{2+}	Ca^{2+} chelate	Phytic or citric acids can sequester Ca^{2+} from pectate and thereby cause softening of canned fruit
Amylose	Crystallization	Alignment of linear starch chains can form insoluble precipitates that contribute to processes like bread staling
Methyl-methionine	Dimethylsulfide	Thermal degradation of sulfur-containing compounds gives rise to many important flavor compounds
Hydrogen sulfide	Discoloration	Iron sulfide appears as black spots both on the can and in potatoes
Fe^{2+}	Fe^{3+}	Increased lipid oxidation and reduced iron absorption are two important consequences that can occur when iron is oxidized
Organic acids	"Detinning"	Coating of cans with enamels greatly reduces solubilization of metal by acid
Anthocyanin, SO_2	Decolorization	The addition of SO_2 to the 4-position of anthocyanins to form a bisulfite addition product causes decolorization

17.6.4 Changes in Molecular Structures

Chemical reactions during processing are influenced in many ways other than just cell disruption. Thermal processing denatures proteins and alters the interaction of proteins with lipids, water, and so forth. Inactivation of enzymes is essential for producing foods with long shelf lives. Also, membrane structures such as chloroplasts are irreversibly altered by heat processing and the components

(e.g., chlorophyll, lipids, and proteins) become more available to participate in reactions. Destruction of the native environment surrounding pigments results in color changes.

Polysaccharides in plant cells are highly ordered in their native state and the structural integrity of the cells is dependent on this order. Heating generally causes these large molecules to absorb water, to "swell," and to become more mobile and more available to participate in chemical reactions. Swelling may also disrupt the structure enough to cause changes in adjacent components. Starch granules are a familiar example of this, but cell walls undergo similar changes, although to a much lesser extent.

17.6.5 Other Factors Influencing Reaction Rates

In addition to structure-related changes discussed earlier, thermal processing has a wide range of effects on individual components, and these effects depend on the time and temperature of processing and the activation energies of the individual reactions. Examples of these reactions are discussed throughout this book. Drying of plant tissues causes an increase in concentration of solutes, as well as migration of solutes, and depending on the type of the drying process utilized, may expose the tissue to high temperatures and oxygen. All these changes favor increased rates of chemical reactions. Furthermore, migration and concentration of solutes can have a profound effect on macromolecules and dramatically alter the rehydration behavior of dried plant food products.

17.7 CONCLUSION

As the world population increases and utilizable agricultural lands diminish, the problems of food availability are certain to increase and botanical sources of food will tend to supplant animal sources. Furthermore, increased emphasis on consumption of plant tissues for general and specific health reasons is likely to substantially increase the demand for foods as derived from plants. There are many gaps in our knowledge regarding postharvest physiological attributes of edible plant tissues and continued research is needed to improve our ability to better utilize postharvest crops and to deliver the highest quality plant foods to consumers. Totally new approaches to plant food production, quality improvement, and preservation will also evolve from basic research on plant physiology. Recombinant DNA technology offers the potential to dramatically improve the quality of many plant products, but such advances require a good understanding of the biochemical determinants of quality.

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18 Impact of Biotechnology on Food Supply and Quality

Martina Newell-McGloughlin

CONTENTS

18.1 INTRODUCTION

The agri-food industry in the next decade will operate in a rapidly changing world environment due to increased competitiveness, globalization of prices, and consumer demands for food quality, safety, health enhancement, and convenience. The food industry in the United States is the country's largest manufacturing industry with revenues exceeding 500 billion dollars per year. The key research priorities of this industry that are targeted for the next decade include:

1. Increase processing efficiencies with a reduction of environmental impact.
2. Expand development of value-added technologies.
3. Understand and utilize component interactions in formulated food systems.
4. Develop and promote strategies to control food borne illnesses.
5. Increase development of foods that promote health and well being.

The set of tools provided through biotechnology has introduced a new dimension to agricultural and food production innovation. Agricultural biotechnology offers the potential to increase food production, improve food quality and healthfulness, reduce the dependency of agriculture on chemicals, alleviate biotic and abiotic stress, and lower the cost of raw materials, all in an environmentally sustainable manner.

Although the scope of biotechnology's influence in the food industry is broad, there are four principal areas that the tools of this technology have potential for major impact and they will be the prime focus of this chapter. They are crop and animal agriculture, bioprocessing, and diagnostics. The development and application of these technologies within the broader legal and socioeconomic context of the food and feed industry will also be discussed.

18.2 BIOTECHNOLOGY IN CONTEXT

In the simplest and broadest sense, biotechnology is a series of enabling technologies, which involves the manipulation of living organisms or their subcellular components to develop useful products and processes. In a time before recorded history, people discovered that microorganisms could be used in food production such as in fermentation processes, to make bread, brew alcohol, and produce cheese. Through mutation and selection processes, use of microorganisms as process tools became more sophisticated as time went by, and this ability took on another dimension with the advent of recombinant DNA (rDNA) technology in the early 1970s. Likewise, humans have been modifying animals and crop plants through cross breeding, selection, and even culling those with undesirable characteristics

for hundreds if not thousands of years. The manipulation of living organisms is one of the principal tools of modern biotechnology. Our capacity to manipulate the genetic makeup of living organisms with complexity and precision has become one of the cornerstones of modern biotechnology. It enables us to enhance the ability of an organism to produce a particular chemical product (e.g., penicillin from fungus), to prevent it from producing a product (e.g., ethylene in plant cells), or to enable an organism to produce an entirely new product (e.g., chymosin in microorganisms).

Many of the products we eat are, or can be, made using the tools of biotechnology. It is possible to enhance the nutritional content, texture, color, flavor, growing season, yield, disease, or pest resistance, and other properties of agricultural crops. Transgenic techniques can be applied to farmed animals to improve their growth, fitness, and other qualities. Enzymes produced using rDNA methods are used to make cheese, keep bread fresh, and produce fruit juices and wines.

18.3 BRIEF HISTORICAL PERSPECTIVE ON CONVENTIONAL FOOD PRODUCTION AND CROP DEVELOPMENT

Ever since the Natufians of the Jordan River valley decided to plant the seeds of either wild barley (*Hordeum spontaneum*) called einkorn or "one grain" (*Triticum monococcum* L. ssp. *boeoticum*), farming had arrived and our "natural" interaction with the plant kingdom ceased to exist. This "neolithic revolution" led to the transition from a hunter-gatherer society to a permanently settled society, which allowed farmers to cultivate, guard, and harvest crops. We have since been manipulating crop plants and later animals using more and more sophisticated technologies through the centuries. For example, modern day maize bears little resemblance to its prime donor ancestor, a relative of teosinte, although probably only three major mutations provided the selective advantage that made it attractive to harvesters but would have been detrimental to survival in the wild. There is a view that the present day rDNA-engineered organisms pose new or greater dangers to the environment or human health. This view, however, is neither supported by the weight of scientific research nor by a great majority of the scientific community.

From a scientific perspective neither the term "genetically modified organism" (GMO) nor the term "living modified organism" (LMO) is an accurate descriptor of the products of modern biotechnology as virtually all domesticated crops and animals have been subjected to varying degrees of modification (Figure 18.1). During the past century, plant and animal breeders expanded the tools of genetic manipulation beyond conventional cross breeding to use a variety of other breeding techniques. In the case of plants, these tools include aneuploidy, diploidy, embryo rescue, protoplast fusion, somaclonal selection, anther culture, and mutation breeding with either radiation (^{60}Co) or ethyl methanesulfonate [9]. Although modern bread wheat *Triticum aestivum* is a "natural" hexaploid made up of three genomic sources that were selected by ancient breeders, its pasta cousin tetraploid durum wheat has been subjected to irradiation mutagenesis to select for random modifications in starch and gluten properties for better pasta-making properties. Likewise, most Asian pears on the market today were selected from an irradiated source that conferred black spot fungal resistance presumably by disrupting a gene that played an important role in the pathogenic processes.

The above techniques allow no control at the genome level as plants contain tens of thousands of genes, and the process of mixing and sorting them during breeding is random, imprecise, and uncontrollable. In addition, traditional breeding programs are time consuming, labor intensive, and limited to transfers of genes between closely related species. Identification of desirable progeny carrying the trait gene relies heavily on selection schemes and often requires 7–12 generations to remove unwanted effects. Plants created by these conventional phenotypic selection techniques undergo no formal food or environmental safety evaluation prior to introduction into the environment and marketplace. At another level entirely, the massive accumulation of sequencing data shows extensive genetic similarity between genomes of organisms that are only remotely related. For example, parts

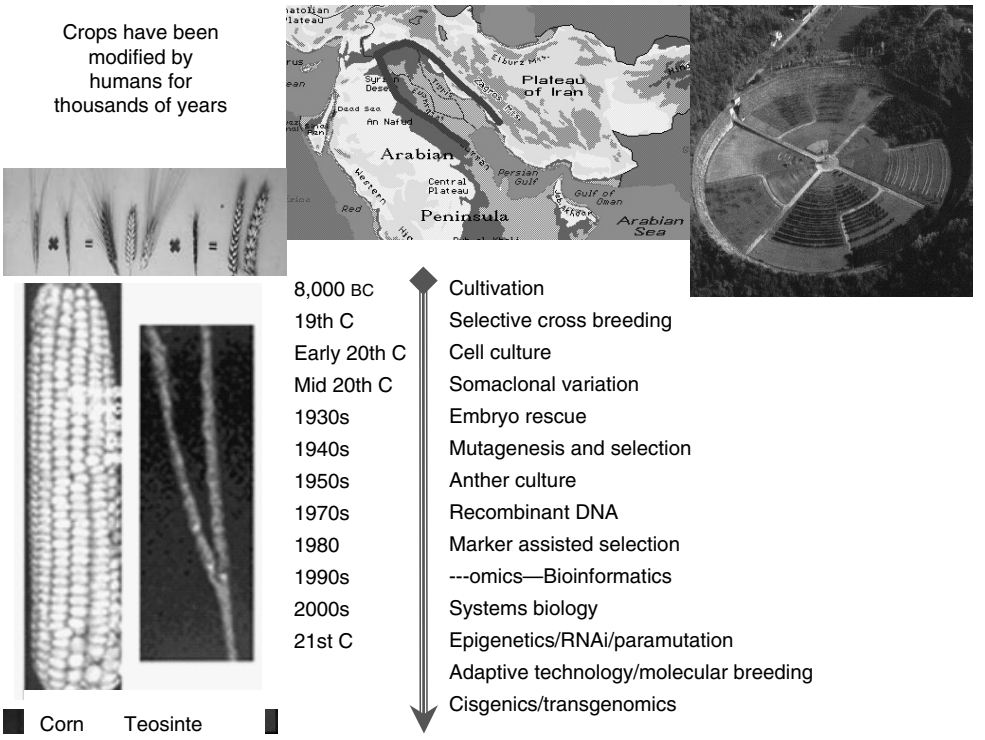


FIGURE 18.1 The map depicts where agriculture and civilization is presumed to have originated in the fertile crescent of Mesopotamia between the Tigris and Euphrates rivers that span current day Iraq. Tetraploid and hexaploid wheat has been traced to this region using genomic and anthropological analysis. Using similar systems modern maize traces its ancestory to teosinte a wild species that grows in meso America. The image on the right shows a modern radiation breeding facility in Japan. The timeline depicts the many technologies that have been applied to modify crops over this period. The later part depicts speculative technologies for future applications.

of the nucleic acid sequence of *Escherichia coli* are identical to that of organisms such as oilseed rape, amphibians, birds, grasses, and mammals—including humans. Such findings put in doubt the value of assigning genes to a particular species. Thus, shuffling of desirable genes from one species to another using rDNA technology need not pose a danger to the environment and public health.

18.4 OVERVIEW OF TECHNOLOGY

Most of the fundamental technologies that fall within the broad rubric of biotechnology are well known. They are polymerase chain reaction (PCR); microarrays; ribozymes; RNA silencing; genomics, proteomics, metabolomics, and bioinformatics; and marker-assisted selection. Before focusing on these technologies, a brief description of a few of the more fundamental tools of biotechnology research is appropriate.

18.4.1 Recombinant DNA Technology

The steps involved in rDNA technology are as follows: (1) to identify the gene that directs the production of the desired substance, (2) to isolate the gene using restriction enzymes, (3) place the gene with appropriate expression signals on a suitable DNA molecule (vector) for transformation, and (4) transfer of the recombined DNA into the appropriate host organism. The final step is to select the transformant that has the desirable characteristics and "clonally" reproduce it.

Cloning *in vivo* can be done in unicellular prokaryotes like *E. coli*, in unicellular eukaryotes like yeast, and in plant or mammalian cells grown in tissue culture. In every case, the rDNA must be taken up by the cell in a form in which it can be replicated and expressed. This is achieved by incorporating the DNA into a vector.

A number of modified viruses can serve as vectors. However, in microbes, cloning is normally done using a plasmid, or what is termed a bacterial artificial chromosome, that can accommodate longer sequences, as the vector. Plasmids are molecules of DNA that are found in bacteria separate from the bacterial chromosome. They are small (a few thousand base pairs), usually carry only one or a few genes, are circular, and have a single origin of replication. Plasmids are replicated by the same machinery that replicates the host bacterial chromosome. Some plasmids are copied at about the same rate as the chromosome (see Figure 18.2), so a single cell is apt to have only a single copy of the plasmid. Other plasmids are copied at a high rate and a single cell may have 50 or more of them.

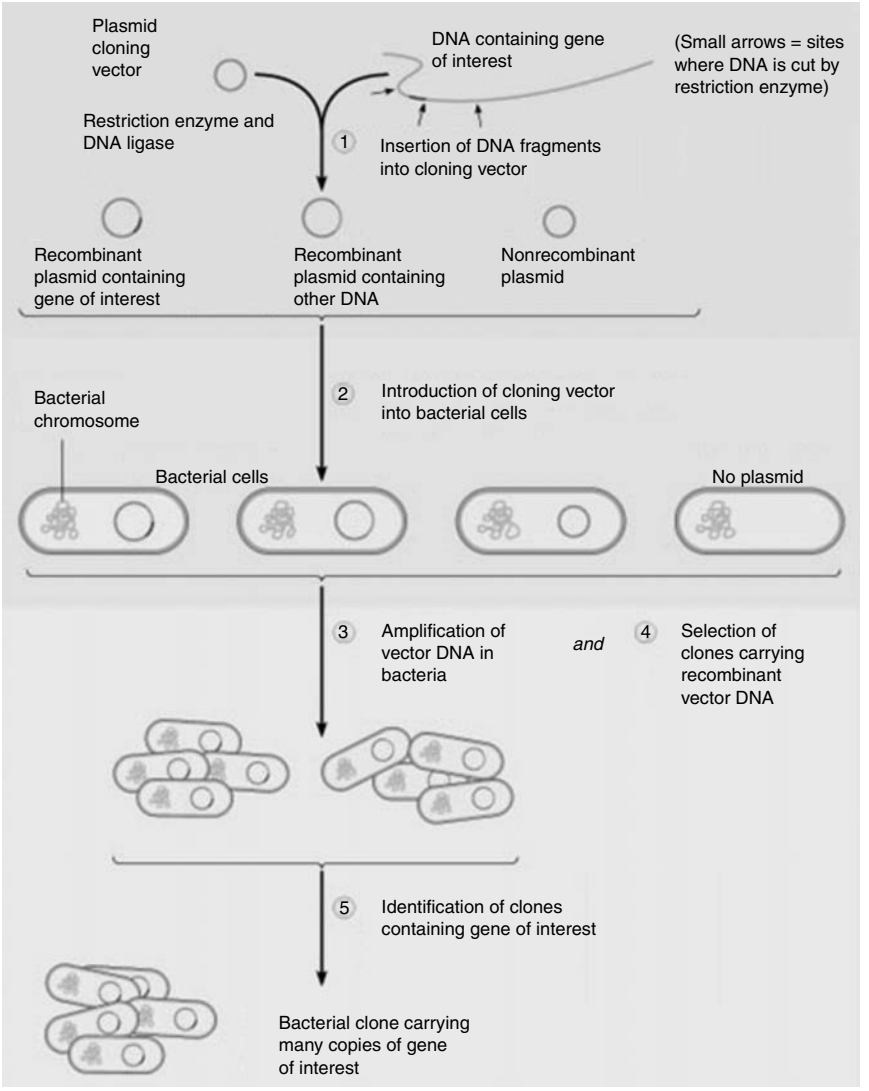


FIGURE 18.2 Plasmids enter the bacterial cell with relative ease. This occurs in nature and may account for the rapid spread of antibiotic resistance in hospitals and elsewhere. Plasmids can be deliberately introduced into bacteria in the laboratory transforming the cell with the genes that have been spliced into the plasmid.

Genes on plasmids with high numbers of copies are usually expressed at high levels. In nature, these genes often encode proteins (e.g., enzymes) that protect the bacterium from one or more antibiotics and in many instances carry virulence and toxin-producing cassettes of genes.

18.4.2 Polymerase Chain Reaction

Polymerase chain reaction remains the dominant technique amongst those that amplify target DNA such as for the detection of a food contaminant. In the PCR two short, chemically synthesized DNA fragments, called primers, are hybridized to the DNA that is then amplified through a repeated cycle

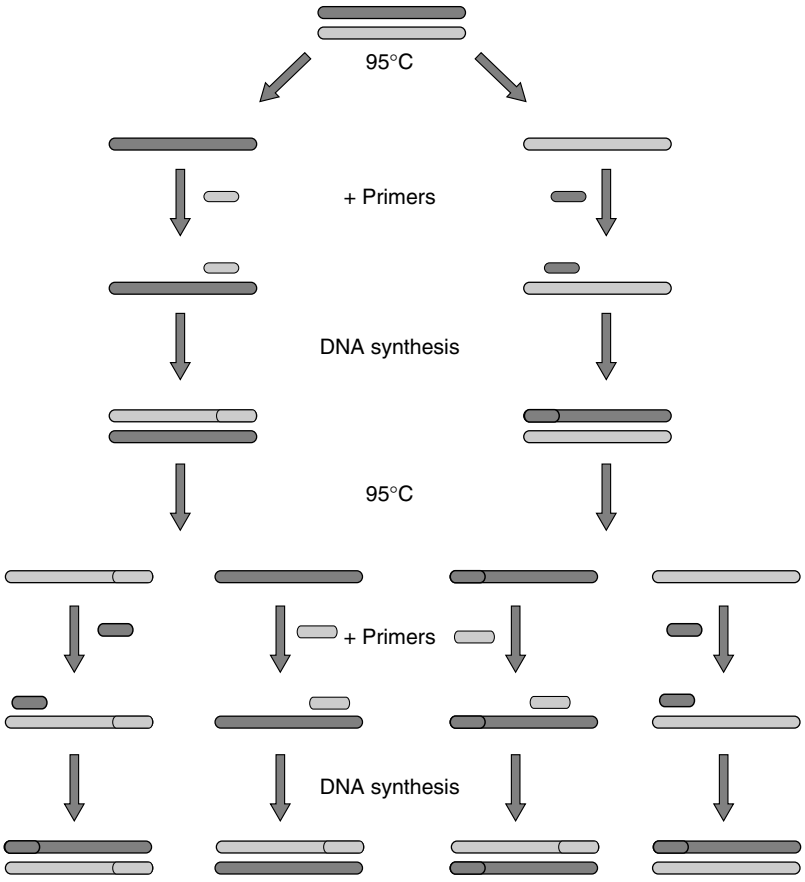


FIGURE 18.3 In order to perform PCR, a portion of the sequence of the DNA molecule to be replicated must be known. Primers are synthesized (short oligonucleotides containing about two dozen nucleotides) that are precisely complementary to the sequence at the 3′ end of each strand of the target DNA. The DNA sample is heated to separate its strands and mixed with the primers. The primers bind to their complementary sequences in the DNA. The reaction mixture must contain all four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), and a heat tolerant DNA polymerase (*Taq* Polymerase). Synthesis precedes $5' \rightarrow 3'$ using the original strand as the template. Polymerization continues until each newly synthesized strand has proceeded far enough to cover the site recognized by the other primer. There are now two DNA molecules identical to the original molecule. The newly synthesized molecules, now act as templates, they are subsequently heated to separate strands, and the process is repeated. Each cycle doubles the number of DNA molecules. Using automated equipment, each cycle of replication can be completed in less than 5 min. After 30 cycles, what began as a single molecule of DNA has been amplified into more than a billion copies ($2^{30} = 1.07 \times 10^9$).

of reactions (Figure 18.3). Vanishingly small amounts of DNA can be amplified to concentrations sufficient for detection by gel electrophoresis and staining. The specificity of both hybridization reactions and PCR can often be adjusted by altering conditions, so that assays have been developed for a single DNA sequence or for a family of related DNA sequences, using the same probe or primers.

18.4.3 Microarrays (Biochips)

In 1991, Biochips were developed for commercial use by Affymetrix. DNA chips or microarrays represent a "massively parallel" genomic technology (Figure 18.4) [67]. They facilitate high-throughput analysis of thousands of genes simultaneously, and are thus potentially very powerful tools for gaining insight into the complexities of higher organisms including analysis of gene expression, detecting genetic variation, making new gene discoveries, fingerprinting strains, and developing new diagnostic tools. These technologies permit scientists to conduct large-scale surveys of gene expression in organisms, thus adding to our knowledge of how they develop over time or respond

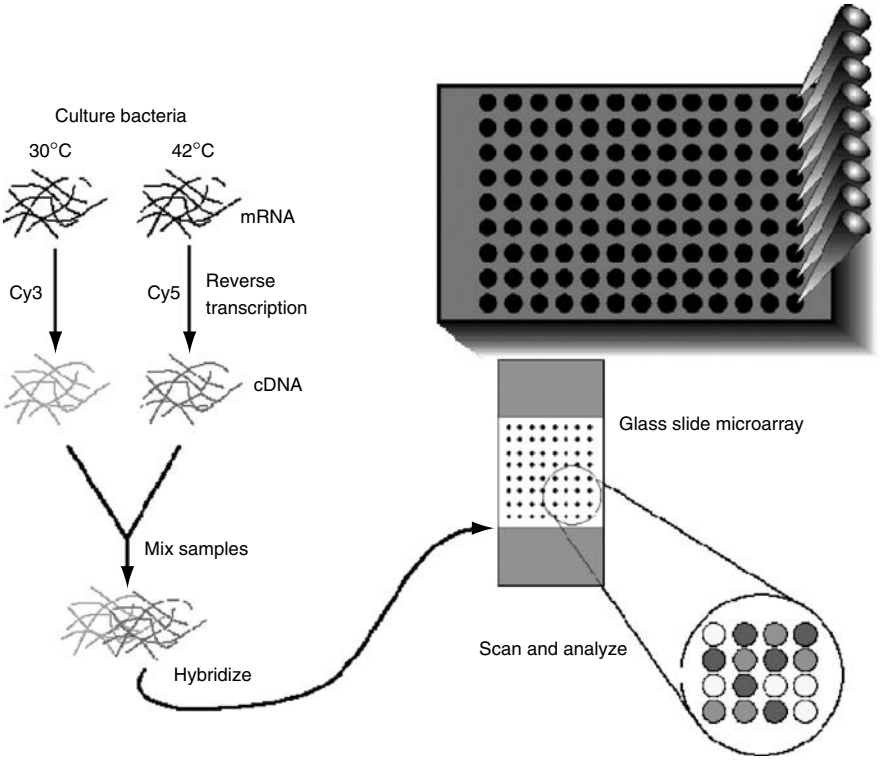


FIGURE 18.4 Microarray Technology. Microarray technology allows the simultaneous examination of tens of thousands of genes through the use of slides or chips. The technology involves extracting all mRNA from the target cells, converting it to its cDNA, labeling it with a fluorescent dye, and applying it to a microarray. The different molecules of DNA attach to their corresponding genes. The same procedure is done to a control group of cells, but with a different color of fluorescent dye. A laser scans the microarray and analyzes the intensity of the different colors to give information on each gene. If a particular gene is very active, it produces many molecules of mRNA that hybridize to the DNA on the microarry and generate a very bright fluorescent area. Genes that are somewhat active produce fewer mRNAs, which results in dimmer fluorescent spots. If there is no fluorescence, none of the messenger molecules has hybridized to the DNA, indicating that the gene is inactive. This technique can be used to examine the activity of various genes at different times.

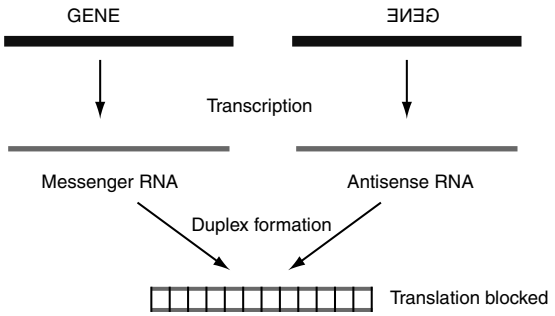


FIGURE 18.5 Antisense technology. RNA can form duplexes just as DNA does. GENE represents the transcript of the positive strand. Is mirror image represents the transcript from the complementary DNA strand produces RNA whose sequence of bases is complementary to the first strand. The second strand is called the antisense strand because its sequence of nucleotides is the complement of message sense. When mRNA forms a duplex with a complementary antisense RNA sequence, translation is blocked.

to various environmental stimuli. These techniques are especially useful in gaining an integrated view of how multiple genes are expressed in a coordinated manner. These DNA chips have broad commercial applications and are now used in many areas of basic and clinical research including the detection of drug resistance mutations in infectious organisms, direct DNA sequence comparison of large segments of the human genome, the monitoring of multiple human genes for cancer-associated mutations, the quantitative and parallel measurement of messenger RNA (mRNA) expression for thousands of genes, and the physical and genetic mapping of organisms of interest.

18.4.4 Antisense Technology

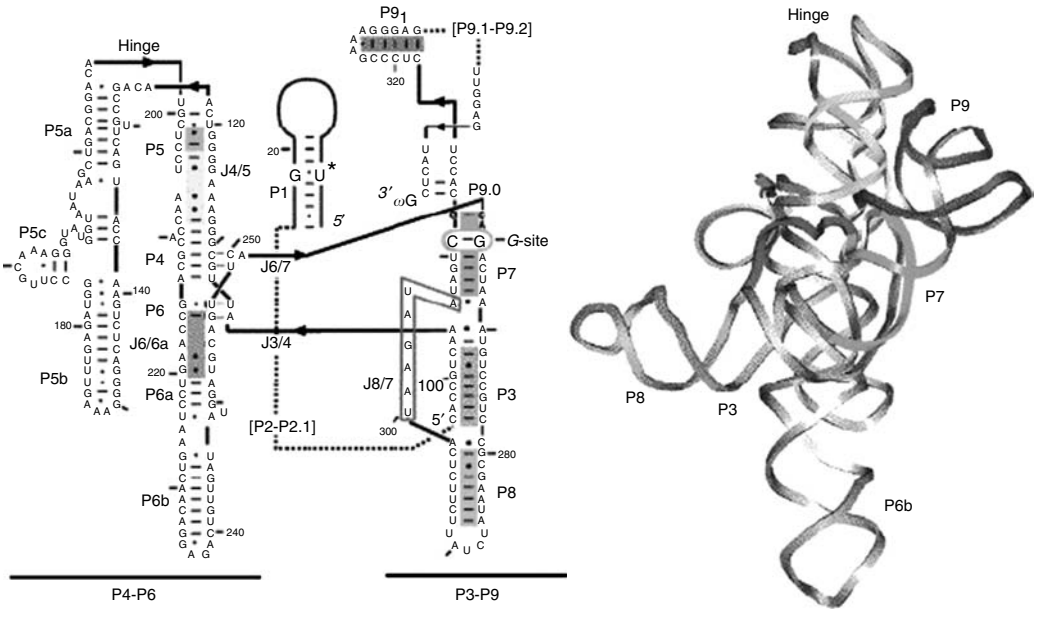
Antisense technology works because nucleic acids have a natural affinity for each other. When a gene coding for the target in the genome is introduced in the opposite orientation, the reverse RNA strand anneals and effectively blocks expression of the enzyme. This technology was patented by Calgene for plant applications and was the technology behind the famous FLAVR SAVR tomatoes (see Figure 18.5).

18.4.5 Ribozymes

Ribozymes are RNA-based enzymes. Although enzymes made up of protein are the dominant form of biocatalyst in modern cells, there are at least eight natural RNA enzymes, or ribozymes, that catalyze fundamental biological processes. It is believed that these ribozymes might be the remnants of an ancient form of life that was guided entirely by RNA. Basically, a ribozyme is a catalytic RNA molecule capable of cleaving itself and other target RNAs and therefore can be useful as a control system for turning off genes or targeting viruses (Figure 18.6) [12]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. More recently, ribozymes have been used for transgenic animal research, gene target validation, and pathway elucidation. However, targeting ribozymes to the cellular compartment containing their target RNAs has proved a challenge.

18.4.6 Riboswitches

Each cell must regulate the expression of hundreds of different genes in response to changing environmental or cellular conditions. The majority of these sophisticated genetic control factors are proteins, which monitor metabolites and other chemical cues by selectively binding to targets.



Mode of action of a ribozyme—the molecular scissors

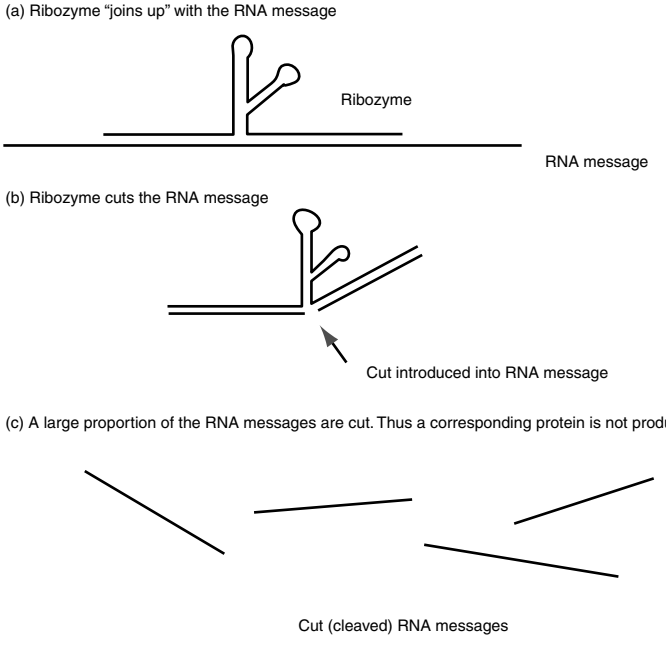


FIGURE 18.6 Mode of action of a ribozyme. Top: A preorganized active site in the crystal structure of the *Tetrahymena* ribozyme. RNA can engage in intramolecular catalysis including self-splicing and in some cases can act as an enzyme. A wide variety of rRNA molecules and some mRNA molecules (chloroplast and mitochondrial) can splice themselves. These molecules are called ribozymes and some of these molecules show other enzymatic functions. (*Source:* Modified from Cech, T. R. 2000. *Science* 289:878–879.)

Mandal et al. [56] have confirmed that RNA can also form precision genetic switches and that these elements can control fundamental biochemical processes.

Riboswitches are a type of natural genetic control element that uses an untranslated sequence in an mRNA to form a binding pocket for a metabolite that regulates expression of that gene. Potentially engineered riboswitches might function as designer genetic control elements.

18.4.7 RNA Silencing

Increased production of desirable characteristics present an appealing image, for example, grains with increased protein content, fruits, and vegetables with enhanced nutritional value, and flowers with deeper colors. It was in pursuit of the latter goal that a most bemusing and ultimately valuable phenomenon was first observed. Although attempting to create "black" petunias as a model for one of the ultimate floriculture aspirations, the "black" rose, Jorgensen et al. [48] of the University of California-Davis attempted to overexpress the chalcone synthesis gene by introducing a modified copy under a strong promoter. The surprising result was white flowers, and many strange variegated variations between purple and white. This was the first demonstration of what has come to be known as post-transcriptional gene silencing (PTGS). Although initially it was considered a strange phenomenon limited to petunias and a few other plant species, it is now one of the hottest topics in molecular biology. RNA interference (RNAi) in animals and basal eukaryotes, quelling in fungi, and PTGS in plants are examples of a broad family of phenomena collectively called RNA silencing [38,68]. In addition to its occurrence in these species it has roles in viral defense and transposon silencing mechanisms among other things. Perhaps most exciting, however, is the emerging use of PTGS and, in particular, RNAi—PTGS initiated by the introduction of double-stranded RNA (dsRNA)—as a tool to knock out the expression of specific genes in a variety of organisms.

So how does it work and how can it be harnessed for functional genomics experiments and to selectively modify gene expression? Instead of producing large quantities of new proteins, high-expressing transgenes (genes from another source) introduced into the plant can actually inhibit the expression of the plant's own genes by triggering sequence-specific destruction of similar transcripts (Figure 18.7) [21]. Thus, the transgene ends up silencing both its own expression and that of similar endogenous genes when the concentration of transgene transcript (mRNA) becomes too high in the cytoplasm. This unintended "RNA silencing" can nonetheless be harnessed by scientists, for example, to eliminate unwanted gene expression, and is used by the plant itself to inhibit protein synthesis by infecting RNA viruses. A role in genome protection is also likely because there is enhanced transposon mobility in RNA silencing—defective mutants of *Chlamydomonas reinhardtii* and *Caenorhabditis elegans* and because DNA transposition is suppressed by RNA silencing in *Drosophila melanogaster* [7,15].

RNA silencing can affect the entire plant, but more often it silences genes in ordered patterns that follow features of plant morphology, believed to reflect underlying prepatterns of transgene transcription. Some patterns, however, suggest that cosuppression per se might not be cell-autonomous, that is, it can be transmitted between cells, perhaps throughout the entire plant.

18.4.8 Protein Engineering

Another extremely promising area of genetic engineering, in the broader sense is protein engineering. New enzyme structures may be designed and produced in order to improve on existing enzymes or create new activities. The principal approaches are (1) site-directed mutagenesis, (2) random mutation and selection, and (3) directed evolution which is a refinement of the latter. However, from a practical point of view, much of the research effort in protein engineering has gone into studies concerning the structure and activity of enzymes chosen for their theoretical importance or ease of preparation rather than industrial relevance. With a greater focus on "green" production system this emphasis is now shifting.

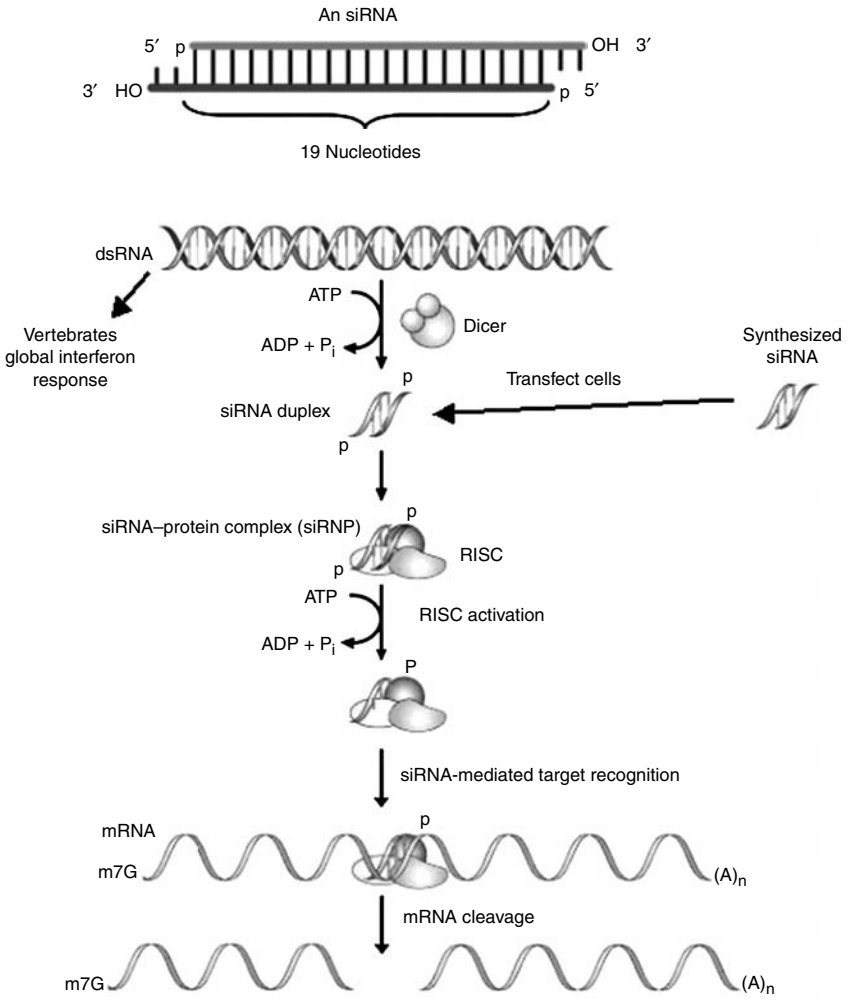


FIGURE 18.7 Schematic diagram of the pathway of RNAi. Once an mRNA is cleaved by RNAi, since the ends of the mRNA are no longer protected by a 5′ cap and a 3′ poly(A) tail, the free ends of the two mRNA fragments are rapidly degraded by cell RNAses; RISC = RNA-induced silencing complex; siRNA = small interfering RNA. (*Source:* Dykxhoorn, D. M., et al. 2003. *Nat. Revs. Mol. Cell. Biol*. 4:457–467.)

The preferred approach for improving enzyme activity and/or specificity and creating new enzymes is by the stepwise substitution of only one or two amino acid residues out of the total protein structure (Figure 18.8 and 18.9) [14]. Although a large database of sequence–structure correlations is available and growing rapidly together with the necessary software, it is presently insufficient to accurately predict three-dimensional changes as a result of such substitutions. The main problem is assessing the long-range effects, including solvent interactions, on the new structure. As the many reported results would attest, the science is at a stage where it can explain the structural consequences of amino acid substitutions after they have been determined but cannot accurately predict them. Protein engineering, therefore, is presently rather a hit or miss process which may be used with only little realistic likelihood of immediate success. Apparently quite small sequence changes may give rise to large conformational alterations and even affect the rate-determining step in enzymatic catalysis. However it is reasonable to suppose that, given a sufficiently detailed database plus suitable software, the relative probability of success will increase over the coming years and the products of protein engineering will make a major impact on industrial enzyme technology.

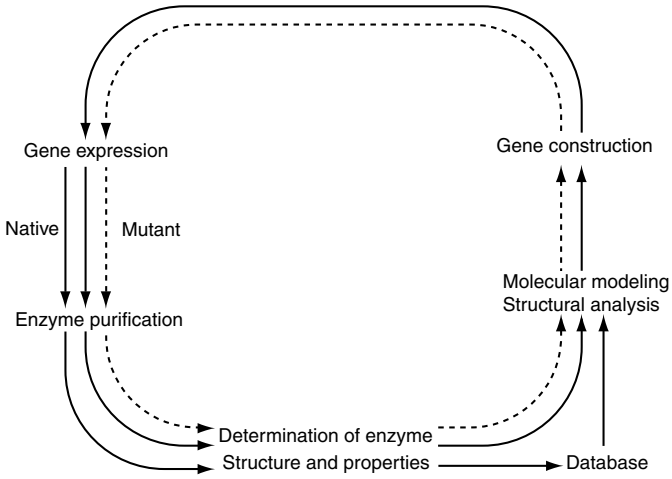


FIGURE 18.8 The enzyme engineering cycle. The process starts with the isolation and characterization of the required enzyme. This information is analyzed together with the database of known and putative structural effects of amino acid substitutions to produce a possible improved structure. This synthetic enzyme is constructed by site-directed mutagenesis, isolated, and characterized. The results, successful or unsuccessful, are added to the database, and the process repeated until the required result is obtained. (*Source:* Chaplin, M. and C. Bucke. 1990. *Enzyme Technology*. Cambridge University Press.)

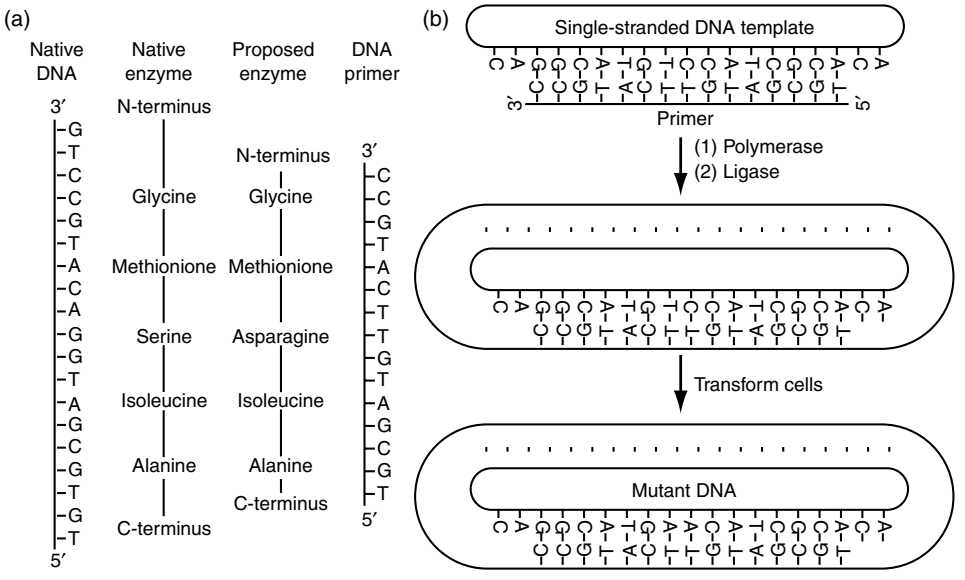


FIGURE 18.9 Site-directed mutagenesis. (a) A short piece of DNA (the primer), complementary to a section of the gene apart from the base mismatch, is synthesized. (b) The oligonucleotide primer is annealed to a single-stranded copy of the gene and is extended with enzymes and nucleotide triphosphates to give a double-stranded gene. On reproduction, the gene gives rise to both mutant and wild-type clones. The mutant DNA may be identified by hybridization with radioactively labeled oligonucleotides of complementary structure. (*Source:* Chaplin, M. and C. Bucke. 1990. *Enzyme Technology.* Cambridge University Press, Cambridge.)

18.4.9 Genomics, Proteomics, Metabolomics, and Bioinformatics

Functional genomics can be defined as establishing a link between gene expression and cellular function. Thousands of genes in a single tissue type vary in levels of expression at different developmental stages, in health and disease, at different chronological times, and in response to environmental variation. Functional genomics provides insight into all the genes involved and the roles they play. Although applications in medicine have been the main thrust of this development, this technology is now having a major impact in agricultural biotechnology, and most specifically plant biotechnology research.

Proteome is the complete set of proteins expressed and modified after their expression from the genome. Proteomics refers to the study of the structure, function, location, and interaction of proteins within and between cells. Proteomics is particularly being driven by global analyses of gene expression and inferences derived from DNA sequence data. However, the study of proteins is not a simple linear extrapolation from knowledge of the DNA sequence. It is a highly complex multidimensional field of endeavor. Each cell produces thousands of proteins, each with a specific function. Proteins differ greatly from one another, even within the same individual, but DNA molecules are remarkably similar. In addition, unlike the unvarying genome, an organism's proteome is so dynamic that an almost infinite variety of protein combinations exist. The genome is constant irrespective of cell type and age, but the proteome varies from tissue to tissue, cell to cell, and with age and time. The cellular proteome changes in response to other cells in the organism and external environmental stimuli. A single gene can code for different versions of a protein, each with a different function. In this area of proteomics, the methods for analysis of protein profiles, and cataloging protein–protein interactions on a genome-wide scale are technically more difficult but improving rapidly, especially for microbes. Functional genomics will impact most areas of biology, from fundamental biochemistry to improvement of quality, and agronomic traits in crops, improved protection against pathogenic microbes, and improved exploitation of beneficial microbes.

18.4.10 Bioinformatics

Sequencing of bacterial, plant, and human genomes has created a vast quantity of data, which are not easily examined or understood. Given the sequence of the human genome, for example, it is an immense task to identify individual genes. Similar problems exist for a wide variety of topics in both structural and functional genomics, primarily due to the scale and parallel nature of these approaches.

The acquisition of relational databases, as well as the development of efficient methods for searching and viewing these data, constitutes a discipline called "bioinformatics." In a broader view, bioinformatics contains computational or algorithmic approaches to the production of information from large amounts of biological data, and this might include prediction of protein structure, dynamic modeling of complex physiological systems, or the statistical treatment of quantitative traits in populations to determine the genetic basis for these traits. Unquestionably, bioinformatics is now an essential component of all research activities utilizing structural and functional genomics approaches for analysis at the sequence level, in structure modeling, and in modeling, linking, and simulating complex higher-level structures such as metabolic and neurological pathways.

The evolving techniques of biotechnology in the postgenomic era can provide the necessary tools to allow individuals to operate at peak levels in the many and varied environments. One of the critical focus areas for optimization of the healthfulness of the individual is through determination (and development) of best-matched diet regimen for the individuals own genetic make up—an area termed nutrigenomics. Nutrigenomics is the modeling the complex interactions between diet and genes focusing on "personalized" nutrition for optimal health and longevity as well as in genotype-based dietary interventions for the prevention, mitigation, or possible cure of susceptible disease types, as measured by the tools of metabolomics. Metabolomics employs analytical techniques that

generate profiles of the metabolites, that is, chemical substances within a biological sample. Unlike targeted analysis, these techniques are indiscriminate in that they do not require prior knowledge of every single substance that is present. Application of the knowledge of individual metabolism will revolutionize the ability of nutrition to deliver health benefits through food in the same way that knowledge of genomics will revolutionize individual treatment of disease with pharmaceuticals. However, this research is still very much at the embryonic stage and the tools and targets are still being elucidated.

18.5 INDUSTRIAL BIOTECHNOLOGY: ENZYMES, FOOD, AND FEED

Industrial biotechnology applies the techniques of modern molecular biology to improve the efficiency and reduce the environmental impacts of processes in industries such as food production, grain cleaning, textiles, paper, and pulp and specialty chemicals.

Enzymes were important agents in food production long before modern biotechnology was developed. They were used, for instance, in the clotting of milk to prepare cheese, the production of bread, and the production of alcoholic beverages. Nowadays, enzymes are indispensable to modern food processing technology and have a great variety of functions. They are used in almost all areas of food production including grain processing, milk products, beer, juices, wine, sugar, and meat.

There are several advantages of using GMOs for the production of enzymes and are as follows:

- It is possible to produce enzymes with a higher specificity and purity.
- It is possible to obtain enzymes which would otherwise not be available for economical, occupational health, or environmental reasons.
- Due to higher production efficiency, there is an additional environmental benefit through reducing energy consumption and waste from production plants.
- For enzymes used in the food industry particular benefits are, for example, a better use of raw materials (juice industry), better keeping quality of a final food and thereby less wastage of food (baking industry), and a reduced use of chemicals in the production process (starch industry).
- For enzymes used in the feed industry particular benefits include a significant reduction in the amount of phosphorus released to the environment from farming.

In almost all cases the enzymes are used as processing aids in food. This means that they only have a function during the food production process. In the final food they are either not present, or they have no function. Over the past 15–20 years, an increasing variety of food enzymes have been produced using GMOs. A good example is the milk clotting enzyme chymosin. Chymosin, also known as rennin, is a proteolytic enzyme synthesized by chief cells in the stomach. Its role in digestion is to curdle or coagulate milk in the stomach, a process of considerable importance in the very young animal. If milk were not coagulated, it would rapidly flow through the stomach and miss the opportunity for initial digestion of its proteins. Chymosin efficiently converts liquid milk to a semisolid like cottage cheese, allowing it to be retained for longer periods in the stomach. Chymosin secretion is maximal during the first few days after birth, and declines thereafter, replaced in effect by secretion of pepsin as the major gastric protease. Chymosin is secreted as an inactive proenzyme called prochymosin that, like pepsin, is activated on exposure to acid. Chymosin is also similar to pepsin in being most active in acidic environments, which makes sense considering its mission.

The majority of milk protein is casein and there are four major types of casein molecules: α_{s1}, α_{s2}, β, and κ. The α- and β-caseins are hydrophobic proteins that are readily precipitated by calcium—the normal calcium concentration in milk is far in excess of that required to precipitate these proteins. However, κ-casein is a distinctly different molecule—it is not calcium-precipitable.

Chymosin proteolytically cuts and converts κ-casein into para-κ-casein and a smaller protein called macropeptide. Para-κ-casein does not have the ability to stabilize the micellar structure and the calcium-insoluble caseins precipitate, forming a curd.

Since calf chymosin is expensive, microorganisms have been modified genetically to yield chymosin that is identical to the enzyme obtained from bovine sources. This can be used to produce better-quality cheese than the fungal or other animal (noncalf) rennets. In 1988, chymosin was the first enzyme from a genetically modified source to gain approval for use in food in the United Kingdom, followed by the United States in 1990, thus placing U.K. approval for the first GMO 2 years ahead of the United States. Three such enzymes are now approved in most European countries and the United States. Chymosin from GMOs behaves in exactly the same way as calf chymosin, but its activity is more predictable and it has fewer impurities. Such enzymes have gained the support of vegetarian organizations and of a number of religious authorities including Kosher and Halal processing. In fact Halal is very logical in their assessment of GMOs stating that it is accepted that GMOs from haram sources would be haram. Indeed chymosin derived from recombinant organisms are held to a higher standard and is subjected to more rigorous tests to ensure its purity. Today about 90% of the hard cheese in the United States and United Kingdom is made using chymosin from genetically modified microbes. It is easier to purify, more active (95% as compared with 5%), and less expensive to produce (Figure 18.10) [60].

In the future, it is predicted that nearly all new food enzymes will be produced by using GMOs. Today up to 90% of the enzymes used in large-scale commercial applications result from the exploitation of rDNA technology. In addition to food industries, enzymes are increasingly being used in chemical synthesis. In contrast to inorganic catalysts such as acids, bases, metals, and metal oxides, enzymes are very specific. In some cases, they limit their action to specific bonds in the compounds with which they interact. Most proteases, for instance, can break down several types of protein, but in each protein molecule only certain bonds will be cleaved depending on which enzyme is used. In industrial processes, the specific action of enzymes allows high yields to be obtained with a minimum of unwanted byproducts.

Enzymes are characterized according to the compounds they act upon. Some of the most common enzymes are proteases, which break down protein; cellulases, which break down cellulose; lipases, which act on fatty acids and oils; and amylases, which break starch down into simple sugars (see Table 18.1 [62] for a sample list of recombinant enzymes on the market).

18.5.1 Enzyme Engineering

Much protein engineering has been directed at subtilisin (from *Bacillus amyloliquefaciens*). The principal enzyme in the Novo Nordisk detergent enzyme preparation, Alcalase, is an engineered subtilisin. This has been aimed at the improvement of its activity in detergents by stabilizing it to elevated temperatures, pH, and oxidant strength. Many substitutions, particularly for the glycine residue (Gly166), have been found to increase the specificity of the enzyme for particular peptide links while reducing it for others. However, a more random approach often leads to a more desirable endpoint. Directed evolution (Figure 18.11) [88] is a general term used to describe various molecular techniques that mimic natural selection and lead to this endpoint. These techniques involve randomly introducing mutations at the genotype level followed by selection for the desired characteristics at the proteome level. Such techniques include chemical mutagenesis, error-prone PCR, incremental truncation, and gene shuffling, among others. The development of such directed evolution stemmed from the observation that new protein characteristics often arise from nonobvious mutations. Consequently rational engineering methods, such as site-directed mutagenesis, are of limited use for protein evolution. Using directed evolution at the genetic level, properties such as stability, specificity, pH, and temperature range of the enzyme has been extended [61].

From a food perspective a good example of the unpredictable nature of protein engineering is given by trypsin, which has an active site closely related to that of subtilisin. Substitution of the

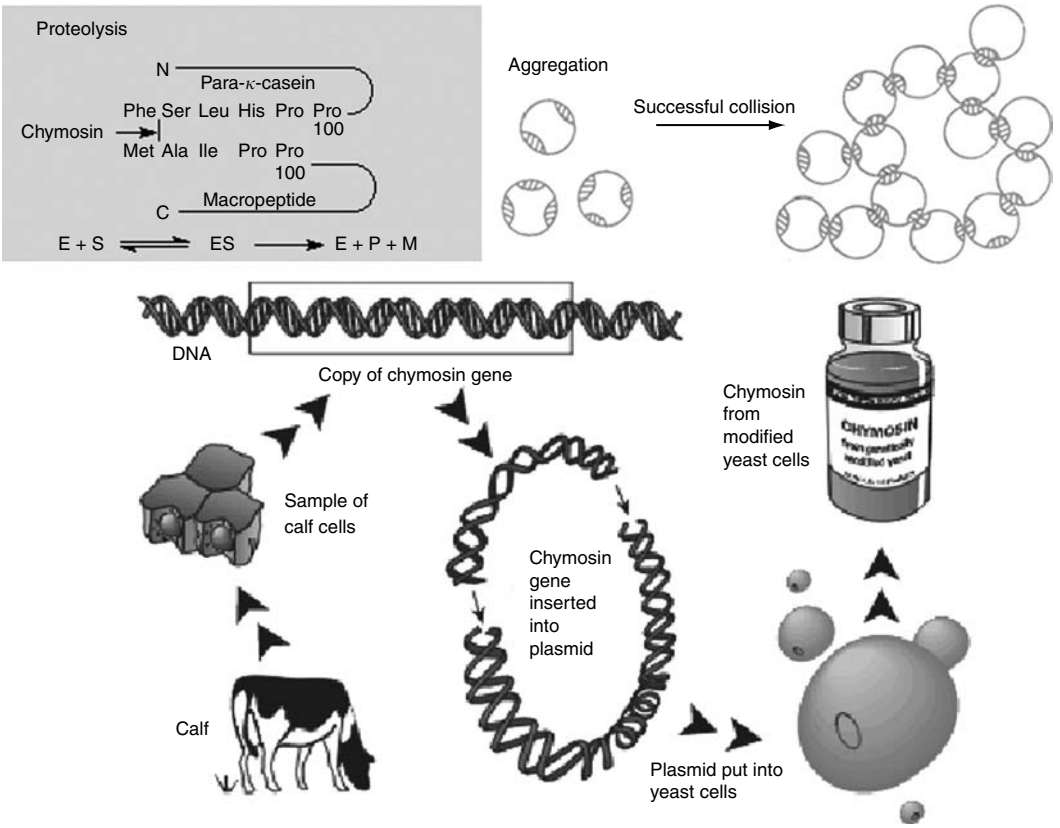


FIGURE 18.10 Recombinant chymosin. Top image: The coagulation of milk by chymosin includes two separate steps: proteolysis and aggregation. Bottom image: The process of genetically modifying microorganisms to yield chymosin that is identical to the enzyme obtained from animals. First the DNA encoding the protein chymosin is isolated from calf cells. A copy of this DNA is spliced into a plasmid and inserted into yeast cells. The plasmid is copied within the yeast cells. Yeast cells are cultivated in a fermenter vessel where they produce chymosin identical to the animal enzyme. As it is composed of a single protein (rather than a mixture) chymosin produced in this way is purer than the rennet which is traditionally obtained from animal stomachs. (*Source:* Modified from NCBE. 2006. The National Centre for Biotechnology Education. http://www.ncbe.reading.ac.uk/NCBE/NCBE/menu.html. Accessed September 25, 2006.)

negatively charged aspartic acid residue at the bottom of its P1 cleft (Asp189), which is used for binding the basic side chains of lysine or arginine, by positively charged lysine gives the predictable result of abolishing the activity against its normal substrates. However, an unexpected result was that this substitution afforded no activity against substrates where the corresponding (S1) sites of the scissile peptide bond were aspartic acid or glutamic acid.

Considerable effort has been spent on engineering more thermophilic enzymes. It has been found that thermophilic enzymes are generally only slightly more stable than their mesophilic counterparts. This may be achieved by the addition of just a few extra hydrogen bonds, an internal salt link or extra internal hydrophobic residues, giving a slightly more hydrophobic core. All these changes are small enough to be achieved by protein engineering. To ensure a more predictable outcome, the secondary structure of the enzyme must be conserved and this generally restricts changes in the exterior surface of the enzyme. For example, by substituting interior glycine or serine residues with alanine one may increase thermostability through small increases in the interior hydrophobicity. It should be recognized that making an enzyme more thermostable reduces its overall flexibility and, hence, it is probable that the synthetic enzyme produced will have reduced catalytic efficiency.

TABLE 18.1
Examples of Engineered Enzymes

Principal Enzymatic Activity	Host Organism (Production Organism)	Donor Organism	Application Examples (Nonexhaustive List)
α-Acetolactate decarboxylase	*Bacillus amyloliquefaciens* or *subtilis*	*Bacillus* sp.	Beverage
α-Amylase	*B. amyloliquefaciens* or *subtilis*	*Bacillus* sp.	Starch, beverage
	Bacillus licheniformis	*Bacillus* sp.	Starch, fruit/vegetable, beverage, sugar, bakery
Catalase	*Aspergillus niger*	*Aspergillus* sp.	Milk, egg
Chymosin	*Aspergillus niger* var. *awamori*	Calf stomach	Cheese
	K. lactis	Calf stomach	Cheese
Cyclodextrin glucanotransferase	*Bacillus licheniformis*	*Thermoanaerobacter* sp.	Starch
β-Glucanase	*B. amyloliquefaciens* or *subtilis*	*Bacillus* sp.	Starch, beverage
	Trichoderma reesei or *longibrachiatum*	*Trichoderma* sp.	Starch, diet
Glucose isomerase	*Streptomyces lividans*	*Actinoplanes* sp.	Starch
	Streptomyces rubiginosus	*Streptomyces* sp.	Starch
Glucose oxidase	*Aspergillus niger*	*Aspergillus* sp.	Egg, beverage, bakery, salads
Hemicellulase	*B. amyloliquefaciens* or *subtilis*	*Bacillus* sp.	Bakery
Lipase	*Aspergillus oryzae*	*Candida* sp.	Fats
		Rhizomucor sp.	Fats
		Thermomyces sp.	Fats, bakery
Maltogenic amylase	*B. amyloliquefaciens* or *subtilis*	*Bacillus* sp.	Starch, beverage, bakery
Protease	*Aspergillus oryzae*	*Rhizomucor* sp.	Cheese
	B. amyloliquefaciens or *subtilis*	*Bacillus* sp.	Meat, fish, starch, beverage, bakery, salads
	Bacillus licheniformis	*Bacillus* sp.	Meat, fish
Pullulanase	*Bacillus licheniformis*	*Bacillus* sp.	Starch
	Klebsiella planticola	*Klebsiella* sp.	Starch, beverage, bakery
Xylanase	*Aspergillus oryzae*	*Aspergillus* sp. and *Thermomyces* sp.	Starch
	Aspergillus niger var. *awamori*	*Aspergillus* sp.	Bakery
	Aspergillus niger	*Aspergillus* sp.	Starch, beverage, bakery
	B. amyloliquefaciens or *subtilis*	*Bacillus* sp.	Starch
	Bacillus licheniformis	*Bacillus* sp.	Starch
	Trichoderma reesei or *longibrachiatum*	*Trichoderma* sp.	Starch, beverage

Source: Newell-McGloughlin, M. and J. Burke. 2000. *Biotechnology: Present Position and Future Potential*, Publishers, Teagasc, Dublin, Ireland.

18.5.2 The Future for Enzymes

A revolution has taken place in biotechnology over the past decade with the growing understanding of gene technology. This has had a big impact on the enzyme industry. Techniques such as genetic engineering have enabled enzyme manufacturers to produce large quantities of almost any enzyme no matter what the source. Protein engineering allows the properties of the enzymes to be adjusted prior to production. These tools mean that enzyme technology is at a very exciting phase of development.

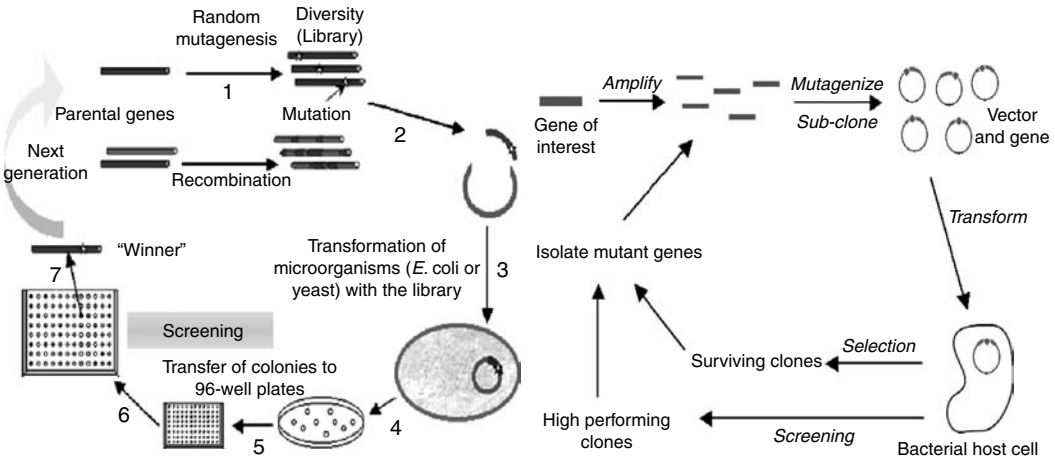


FIGURE 18.11 Directed evolution for enzyme engineering. "Nature" is mimicked by inducing mutations and/or recombinations in the gene which codes for a specific protein. The "fittest" from each generation are selected to go through the next round of evolution. The process is repeated as many times as required, until the optimum biocatalyst is achieved with the desired traits: stability at high temperatures or in organic solvents, better efficiency, improved catalytic activities, and so forth. (Courtesy of Michael Toney, UC Davis.)

The traditional method of enzyme production can be very time consuming. It begins with the hunt for the enzyme having the desired characteristics among microorganisms isolated from soil or water samples taken from around the world. Once an enzyme exhibiting the desired properties is found, the microorganism which produced it is cultured and identified. It is then mutated by chemical or radiological means. This is done to try to obtain such favorable characteristics as high yields and growth rates on simple media and to remove undesirable byproducts. When this has been carried out successfully, the optimum conditions for fermentation have to be determined and toxicity studies run. Finally, permission has to be obtained from the relevant authorities to use the resultant product for large scale industrial purposes.

The advent of genetic engineering, and the knowledge amassed on strains of three types of microorganism—a bacterium (*Bacillus*), a fungus (*Aspergillus*), and a yeast (*Saccharomyces*)—have transformed the nature of industrial enzyme production. These microorganisms are well understood and have proved safe to handle, quick to grow, and capable of producing high yields of enzyme, often by excreting it into the fermentation medium. Another advantage is that the medium in which they grow and perform well is already known, thus minimizing further costly experimentation to optimize fermentation conditions. Today, once a useful enzyme has been identified in a new microorganism, the genetic material coding for its structure can be quickly transferred into one of the suitable host microorganisms mentioned above. With improvements in promoter identification and codon usage the production of the enzymes in ever higher yields is becoming possible. A further benefit of genetic engineering is that enzymes can now be produced with a much higher degree of purity and free from contaminating enzyme activity. This allows for greater control over processes catalyzed by enzymes and, in turn, leads to higher quality end-products.

There is a rapidly growing interest in the industrial use of enzymes for synthesizing organic chemicals. Enzymes can either be used to build more complex molecules from simple ones or to selectively break down a mixture of larger molecules. For example, specific glycosyltransferases called cyclodextrin glycosyltransferases can be used to create cyclodextrins from simple starch molecules. These cyclodextrins are useful carriers of certain sensitive or labile compounds such as vitamins and flavors. No chemical means exists for creating this class of substances, so the enzymatic route is unique.

A number of possibilities now also exist for the construction of artificial enzymes. These are generally synthetic polymers or oligomers with enzyme-like activities, often called synzymes. They must possess two structural entities, a substrate-binding site and a catalytically effective site. It has been found that producing the facility for substrate binding is relatively straightforward but catalytic sites are somewhat trickier. Both sites may be designed separately but it appears that, if the synzyme has a binding site for the reaction transition state, this often achieves both functions. Some synzymes are simply derivatized proteins. An example is the derivatization of myoglobin, the oxygen carrier in muscle, by attaching ruthenium amine probes to three surface histidine residues. This converts it from an oxygen carrier to an oxidase, oxidizing ascorbic acid (vitamin C) while reducing molecular oxygen. The synzyme is almost as effective as natural ascorbate oxidases. It is impossible to design protein synzymes from scratch with any probability of success, as their conformations are not presently predictable from their primary structure. Such proteins when eventually designed can also be used to demonstrate the drawbacks of natural enzymes, being sensitive to denaturation, oxidation, and hydrolysis.

As with every other biotech field the rapidly advancing technologies of functional genomics, bioinformatics, domain shuffling, and so forth are providing new tools for the rapid identification of new targets for industrial enzymes research.

18.6 AGRICULTURAL CROPS

18.6.1 Plant Crops, Horticulture, Food, and Feed

In agriculture, biotechnology in the form of rDNA technology is a powerful assistant to traditional plant and animal breeding. Traditional breeding programs are time consuming, labor intensive, and limited to transfers of genes between closely related species. In addition, because the breeder has no control at the genome level, many undesirable traits can also be incorporated, such as lower yield and slower growth. rDNA technology permits the precise and predictable manipulation of genes. Single traits can be modified much more quickly than was possible using traditional selection and breeding methods alone.

18.6.1.1 Transformation

The key tool for all applications of plant biotechnology whether it be basic research or practical applications is plant transformation (Figure 18.12). There are verified methods for stable introduction of novel genes into the nuclear genomes of over 120 diverse plant species. Important considerations for transformation are (1) the biological and practical requirements for transformation systems; (2) the integration of optimum explant sources, tissue culture, gene transfer, selection, and transgene expression strategies to achieve transformation in recalcitrant species; and (3) transformation validation. The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 [42]. Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector has been extended to over 140 species in at least 35 families. Successes include most major economic crops, vegetables, and ornamental, medicinal, fruit, tree, and pasture plants. The rapid and simultaneous developments in transformation technology and information technology make tabulations of transformed species quickly out of date and it is advisable to use computer-based searches to locate references to current transformation methods for species of interest. The process of diversification and refinement of transformation techniques for greater convenience, higher efficiency, broader genotype range, and desired molecular characteristics of transformants will continue to good effect for some time. However, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species. Attention is increasingly being directed to achieving the desired patterns of expression of introduced genes and to solving economic constraints on practical plant molecular improvement.

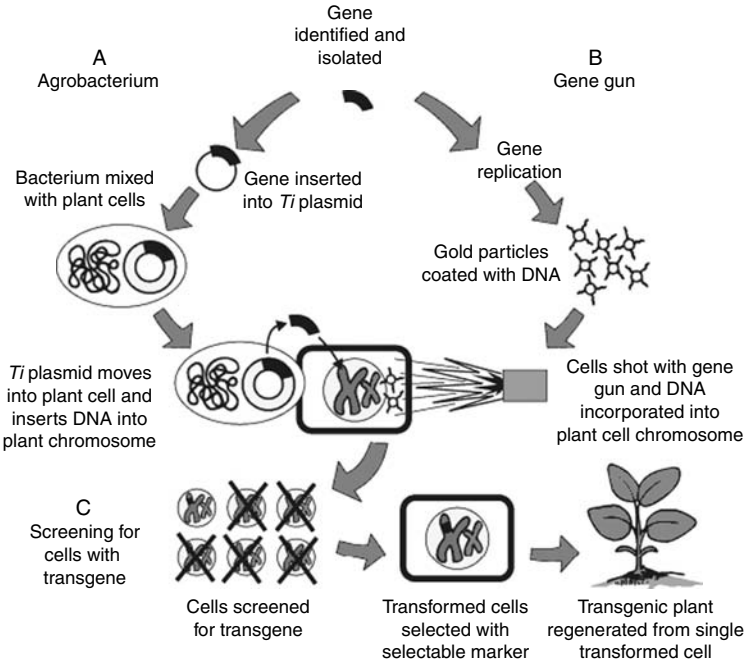


FIGURE 18.12 Steps involved in transformation of plant cells. (Courtesy of Kent Bradford, UC Davis.)

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plants causing the formation of the crown gall tumors. The first evidence indicating this bacterium as the causative agent of the crown gall dates back over 90 years. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease. The initial results of the studies on T-DNA transfer process to plant cells demonstrated three important facts for the practical use of this process in plant transformation. First, the tumor formation is a transformation process of plant cells resulting from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Second, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Third, any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no matter what the source of the DNA. Expression of the gene of course depends on the presence of a suitable promoter.

Since that seminal transformation of tobacco in the early 1980s, great progress in understanding the Agrobacterium-mediated gene transfer to plant cells has been achieved. However, *A. tumefaciens* naturally infects only dicotyledonous plants and many economically important plants, including the cereals, remained inaccessible for genetic manipulation because of lack of effective transformation techniques until the early 1990s. For these cases, alternative direct transformation methods have been developed such as polyethyleneglycol-mediated transfer microinjection, protoplast, and intact cell electroporation and the biolistic gun technology. However, Agrobacterium-mediated transformation has remarkable advantages over direct transformation methods. It reduces the copy number of the transgene, potentially leading to fewer problems with transgene cosuppression and instability. In addition, it is a single-cell transformation system that does not result in the formation of mosaic plants, which are more frequent when direct transformation is used.

The barriers to the transformation of monocots were overcome when, in 1989, a universal procedure was developed for introducing DNA into cells. The method known as Biolistics uses a

"particle gun" to shoot metal particles coated with DNA into cells. Initially, a gunpowder charge subsequently replaced by helium gas was used to accelerate the particles in the gun. There is a minimal disruption of tissue and the success rate has been extremely high for applications in several plant species [76].

Agrobacterium rhizogenes, a causal bacterium of hairy root disease on dicotyledonous plants, harbors a large plasmid called root-inducing (Ri) plasmid. During infection, a portion of the plasmid called T-DNA is transferred from the bacterium to plant cells and is then integrated into a plant chromosomal DNA, by the same mechanism as that known in Ti-plasmid. After the integration of the T-DNA, several genes responsible for hairy root induction are expressed, and the cells then differentiate into adventitious roots. Hairy root cultures grow rapidly, show plagiotropic root growth, and are highly branched on hormone-free medium. Many dicotyledonous plants are susceptible to *A. rhizogenes* and plants have been regenerated from hairy root cultures in a wide range of species. These plants, however, often exhibit an altered phenotype due to the expression of the rol (root locus) loci from the T-DNA. Such abnormalities include reduced apical dominance, reduced fertility, shortened internodes, wrinkled leaves, late flowering, and plagiotropic roots.

A. rhizogenes can also transfer the T-DNA of binary vectors in trans (supplied separately), thereby enabling the production of transgenic plants containing other foreign genes carried on a second plasmid. This method has been used to produce transgenic plants in several species. If there are independent insertions of the Ri T-DNA and binary vector T-DNA, segregation of the separate insertions in subsequent plant generations can produce phenotypically normal transgenic plants, as demonstrated in tomato, tobacco, and oil seed rape.

The application of hairy roots, that is, plant roots formed from plant cells after transformation by *A. rhizogenes*, show promise for the production of bioactive compounds. Transformed root cultures have been established from numerous species of dicotyledonous plants. The possible use of hairy root cultures for the overproduction of secondary metabolites and biotransformation of chemicals is one of the principle foci for research with this system.

18.6.1.2 Input/Agronomic Traits

The first generation of transgenic crops focused largely on input agronomic traits; the next generation will focus more on value-added output traits. During this early phase of the plant biotechnology revolution, the benefits have been largely confined to farmers, who have been able to increase their production, reduce input costs, use less insecticide, increase insect and weed control in an environmentally managed way, enhance conservation tillage, and increase their economic return [32]. Consumers are largely unaware of any benefits to them from this first generation of agricultural biotechnology. For example, it is largely unknown that the level of fumonisin mycotoxin contamination of maize has been reduced by up to 93% with the reduction in insect damage, and therefore decreased fungal spore infections, realized by the introduction of European Corn Borer-resistant Bt maize [59]. This reduction in fumonisin levels has direct safety benefits to humans and animals because those mycotoxins are some of the most noxious substances on crops, resulting in ailments from liver cancer to brain damage. Table 18.2 lists transgenic crops with improved agronomic traits relevant to improvements in food quality, functionality, handling, and utilization. North America remains the epicenter of R&D on plant biotech. Thousands of field trials have been conducted in the two countries. Canada has produced, approved, or field tested more field crops than any other country. The United States has approved in total 15 crops to date, including corn, cotton, canola, soybeans, chicory, cotton, flax, melon, papaya, potatoes, rice, squash, sugar beets, tobacco, and tomatoes.

Agricultural biotechnology has helped farmers around the world boost their productivity and grow crops in more ecologically healthy fields while allowing much more efficient use of resources (Figure 18.13) [44]. This technology allows reduced tillage, which cuts down on greenhouse as emissions, water runoff, machinery use, and soil erosion.

TABLE 18.2
Biotech Crop Plants Intended for Food or Feed That Have Completed All Recommended or Required Reviews for Planting, Food, or Feed Use in the United States

Record Number	Common Name (*Scientific Name*)	Trait Category	Applicant(s)	Event(s)	Trait Description(s)	Reviewed Uses within the United States
1.	Tomato (Lycopersicon esculentum)	Delayed fruit ripening	DNA Plant Technology Corporation	1345-5	Delayed fruit ripening; suppression of endogenous amino cyclopropane carboxylic acid synthase (ACCS)	Planting, food, and feed
2.	Canola *Brassica napus*, *B. napus* var. *napus*	Altered fatty acids and oils	Calgene Inc.	pCGN3828-212/86-18 pCGN3828-212/86-23	High laurate oil; 12:0 acyl carrier protein thioesterase; from *Umbellularia californica*	Planting, food, and feed
3.	Tomato *L. esculentum*	Delayed fruit ripening	Agritope Inc.	35-1-N	Delayed fruit ripening; *S*-adenosylmethionine hydrolase; from *E. coli* bacteriophage T3	Planting, food, and feed
4.	Tomato *L. esculentum*	Delayed fruit ripening	Monsanto Company	8338	Delayed fruit ripening; 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD); from *Pseudomonas chlororaphis*	Planting, food, and feed
5.	Tomato *L. esculentum*	Delayed fruit ripening	Zeneca & Petoseed	B, Da (added line) F (added line) (3 **events total**)	Delayed fruit ripening; polygalacturonase (PG) gene fragment; from tomato	Planting, food, and feed
6.	Tomato *L. esculentum*	Delayed fruit ripening	Calgene Inc.	Line N73 1436-111	Delayed fruit ripening; antisense polygalacturonase (PG); from tomato	Planting, food, and feed

7.	Tomato *L. esculentum*	Delayed fruit ripening	Calgene Inc.	FLAVRSAVR (32 lines in 4 events), 532, 4109a, 5166 (added lines) (5 **events total**)	Delayed fruit ripening; antisense polygalacturonase (PG); from tomato	Planting, food, and feed
8.	Canola *B. napus*, *B. napus* var. *napus*	Phytate degradation	BASF	MPS961 MPS962 (added line) MPS963(added line) MPS964 (added line) MPS965 (added line) (5 **events total**)	Phytate degradation; Phytase; from *Aspergillus niger*	Food and feed
9.	Cantaloupe *Cumumis melo*	Delayed fruit ripening	Agritope Inc.	A B (added line) (2 **events total**)	Delayed fruit ripening; *S*-adenosylmethionine hydrolase; from *E. coli*	Food and feed
10.	Soybean *Glycine max*	Altered fatty acids and oils	DuPont	260-05	High oleic acid; GmFad2-1 gene; from soybean	Planting, food, and feed
11.	Tomato *L. esculentum*	Delayed fruit ripening	Calgene Inc.	CR3-613 CR3-623 (added line) (2 **events total**)	Delayed fruit ripening; antisense polygalacturonase (PG); from tomato	Planting, food, and feed

Records are consolidated from the database and only those records with obvious food quality, functionality, or handling improvements are included here. Product uses are described in the database as: "food," "feed," or "planting." For each product, the database lists only those uses for which the product has completed all relevant procedures required or recommended by the regulatory agencies. Note that not all of the products in the database have completed reviews for all uses.

"Food" use means that a product has completed an FDA review for consumption by humans and if the product is a plant that contains a plant-incorporated protectant (PIP), the PIP has completed EPA reviews.

"Feed" use means that a product has completed an FDA review for consumption by animals and if the product is a plant that contains a plant-incorporated protectant (PIP), the PIP has completed EPA reviews.

"Planting" use means that a product has completed USDA-APHIS review for cultivation in the United States and if the product is a plant that contains a plant-incorporated protectant (PIP), the PIP has completed EPA reviews.

Source: U.S. Regulatory Agencies Unified Biotechnology Website (http://usbiotechreg.nbii.gov/database_pub.asp).

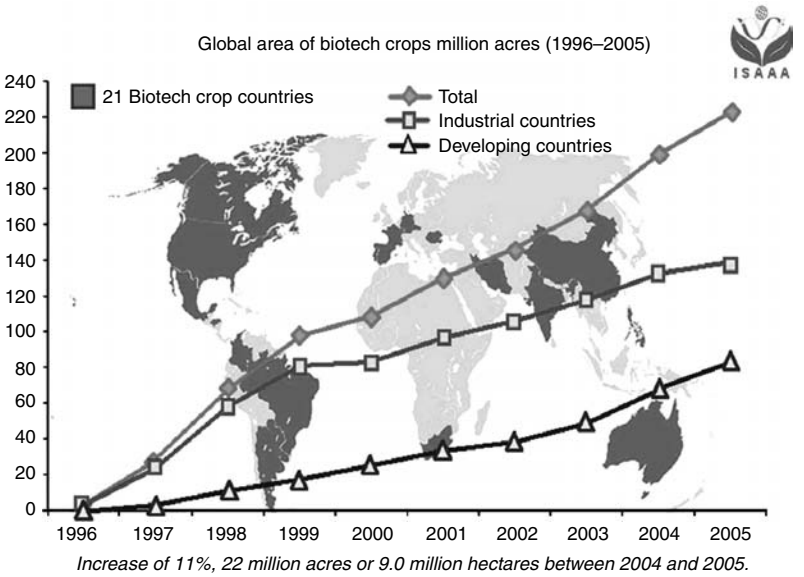


FIGURE 18.13 Global area of biotech crops. (*Source:* James, C. 2006. Global Status of Commercialized Biotech/GM Crops ISAAA Brief No 35 ISAAA, Ithaca, New York. Accessed February 17, 2007.)

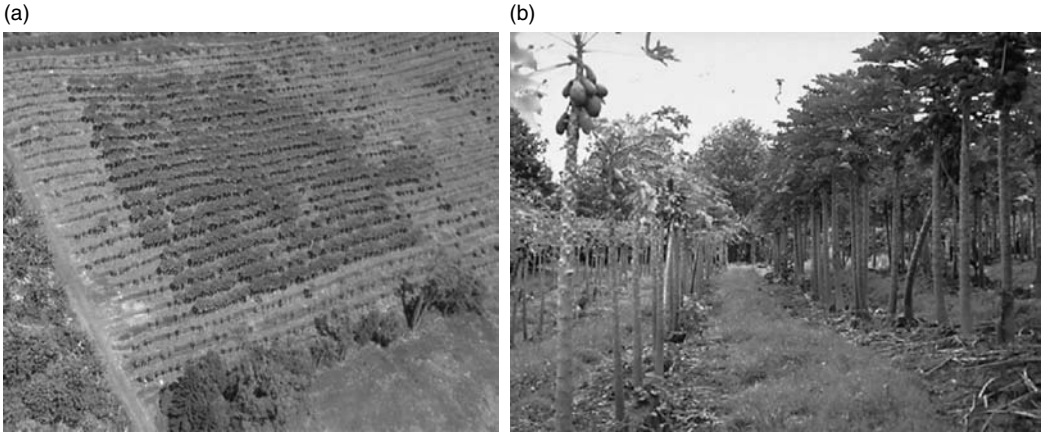


FIGURE 18.14 Field trial of transgenic "UH (University of Hawaii) Rainbow" and "UH SunUp" was established in Puna (India) in October 1995. (a) Aerial view of transgenic field trial. The solid block of green papaya trees are "UH-Rainbow" while the surrounding unhealthy trees are nontransgenic papaya severely infected by PRSV. (b) The progress of the disease caused by PRSV can be clearly seen in rows of nontransgenic papaya (left in picture) as compared to the resistance in rows of "UH Rainbow" (right in picture). Photos courtesy of Steve Ferrara, University of Hawaii.

One of the modified crops cultivated, papaya, is a major tropical fruit crop in Hawaii and the Asian region (Figure 18.14). However, production in many Asian countries is set back by the prevalence of the papaya ringspot virus-resistant (PRSV) disease as well as postharvest losses. The PRSV-resistant papaya, based on RNAi suppression of the coat protein (CP) expression, literally saved the $17 million economy in Hawaii and is of significant importance in Taiwan and other SE Asian countries. CP-based resistance is a demonstration of what is known as PTGS. In addition to protecting conventional papaya plants, by removing the virus reservoir the CP protected papaya

can help organic growers. There have been some claims that CP had sequences in common with a known allergen thus making them allergenic, however, this virus (with a complete intact CP) has been reported on the islands since the 1940s and there have never been any reported cases ever of any allergenic reactions. Even if the CP has modified codons and was presented differently in the transgenic papaya, clinical tests have shown this makes no difference as ingested allergens are sequence specific at the amino acid level while inhaled allergens depend on protein conformation.

This system has now been applied to other species. A 5 year effort to combat plum pox virus disease through PTGS resistance paid off. In 1990, USDA/Agricultural Research Service (ARS) scientists began their efforts with a papaya ringspot virus CP gene obtained from Dennis Gonsalves. This gene shows 70% homology to the plum pox gene and has been used to control other viruses similarly related to papaya ringspot. However, irrespective of the mechanism, it is important that resistance based on a single gene is managed well and alternate control mechanisms are introduced to reduce pressure on the development of viral resistance. Other approaches include expression of the RNA replicating enzymes of the virus, expression of satellite RNA, replicating RNA molecules that are molecular parasites of the virus, or the use of protease inhibitors to interfere with processing of the viral proteins.

The next major phase for agricultural biotechnology is the introduction of traits that provide more readily apparent benefits to the consumer and traits that will confer value-added components from the perspective of the food or feed processor. Challenges that have accompanied GM crops with improved agronomic traits, such as the stalled regulatory processes in Europe, will also affect adoption of nutritionally improved GM products.

18.6.1.3 Processing and Functionality; Safety and Nutrition

Functional foods have been defined as any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. The term nutraceutical is defined as "any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease" [15].

From a health perspective, plant components of dietary interest can be broadly divided into three main categories:

1. Macronutrients (proteins, carbohydrates, lipids, and fiber).
2. Micronutrients (vitamins, minerals, phytochemicals).
3. Antinutrients (substances such as phytate that limit bioavailability of nutrients).
4. Allergens and toxins.

Phytochemicals* and functional food components (often referred to as nutraceuticals) are of increasing interest in the prevention and/or treatment of at least four of the leading causes of death in the United States: cancer, diabetes, cardiovascular disease, and hypertension. The U.S. National Cancer Institute estimates that one in three cancer deaths are diet related and that eight of ten cancers have a nutrition/diet component [6,84]. Other nutrient-related correlations link dietary fat and fiber to the prevention of colon cancer, folate to the prevention of neural tube defects, calcium to the prevention of osteoporosis, psyllium to the lowering of blood lipid levels, and antioxidant nutrients to the scavenging of reactive oxidant species and protection against oxidative damage of cells that may lead to chronic disease, to list just a few [34]. Examples of these phytochemicals are listed in Table 18.3 [15]. One group of phytochemicals, the isothiocyanates (and related glucosinolates, indoles, and sulforaphane) are found in vegetables, such as broccoli, and have been shown to upregulate enzyme systems that block or suppress cellular DNA damage and reduce tumor size [31]. The large

* Phytochemicals are primarily secondary metabolites produced by plants. Plants can produce from 80,000 to 100,000 of these chemicals depending on the plant.

TABLE 18.3
Selected Examples of Plant Components with Suggested Benefit

Class/Components	Source	Potential Health Benefit
Carotenoids		
α-Carotene	Carrots	Neutralizes free radicals that may cause damage to cells
β-Carotene	Various fruits, vegetables	Neutralizes free radicals
Lutein	Green vegetables	Contributes to maintenance of healthy vision
Lycopene	Tomatoes and tomato products (ketchup, sauces)	May reduce risk of prostate cancer
Zeaxanthin	Eggs, citrus, maize	Contributes to maintenance of healthy vision
Dietary fiber		
Insoluble fiber	Wheat bran	May reduce risk of breast and/or colon cancer
β-Glucan	Oats	May reduce risk of cardiovascular disease (CVD)
Soluble fiber	Psyllium	May reduce risk of CVD
Whole grains	Cereal grains	May reduce risk of CVD
Collagen hydrolysate	Gelatin	May help improve some symptoms associated with osteoarthritis
Fatty acids		
Omega-3 fatty acids—DHA/EPA	Tuna; fish and marine oils	May reduce risk of CVD and improve mental, visual functions
Conjugated linoleic acid (CLA)	Cheese, meat products	May improve body composition, may decrease risk of certain cancers
Flavonoids		
Anthocyanidins: cyanidin	Berries	Up-regulates xenobiotic defense enzymes, may reduce risk of cancer
Hydroxycinnamates	Wheat	Antioxidant-like activities, may reduce risk of degenerative diseases
Flavanols: catechins, tannins	Tea (green, catechins), (black, tannins)	Neutralize free radicals, may reduce risk of cancer
Flavanones	Citrus	Neutralize free radicals, may reduce risk of cancer
Flavones: quercetin	Fruits/vegetables	Neutralize free radicals, may reduce risk of cancer
Glucosinolates, indoles, isothiocyanates		
Sulphoraphane	Cruciferous vegetables (broccoli, kale), horseradish	Up-regulates xenobiotic defense enzymes, may reduce risk of cancer
Phenols		
Stilbenes: resveratrol	Grapes	May reduce risk of degenerative diseases; heart disease; cancer
Caffeic acid, ferulic acid	Fruits, vegetables, citrus	Antioxidant-like activities; may reduce risk of degenerative diseases; heart disease, eye disease
Plant stanols/sterols		
Stanol/sterol ester	Maize, soy, wheat, wood oils	May reduce risk of coronary heart disease (CHD) by lowering blood cholesterol levels
Prebiotic/probiotics		
Fructans, inulins, fructo-oligosaccharides (FOS)	Jerusalem artichokes, shallots, onion powder	May improve gastrointestinal health

TABLE 18.3 (Continued)

Class/Components	Source	Potential Health Benefit
Lactobacillus	Yogurt, other dairy	May improve gastrointestinal health
Saponins	Soybeans, soy foods, soy protein-containing foods	May lower LDL cholesterol; contains anticancer enzymes
Soybean protein	Soybeans and soy-based foods	25 g/day may reduce risk of heat disease
Phytoestrogens		
Isoflavones: daidzein, genistein	Soybeans and soy-based foods	May reduce menopause symptoms, such as hot flashes, reduce osteoporosis, CVD
Lignans	Flax, rye, vegetables	May protect against heart disease and some cancers; may lower LDL cholesterol, total cholesterol, and triglycerides
Sulfides/thiols		
Diallyl sulfide	Onions, garlic, olives, leeks, scallions	May lower LDL cholesterol, helps to maintain healthy immune system
Allyl methyl trisulfide, dithiolthiones	Cruciferous vegetables	May lower LDL cholesterol, helps to maintain healthy immune system
Tannins		
Proanthocyanidins	Cranberries, cranberry products, cocoa, chocolate, black tea	May improve urinary tract health May reduce risk of CVD and high blood pressure

Source: Modified from Chassy, B., *et al.* 2004. Safety and Nutritional Assessments of Foods and Feeds Derived Through Biotechnology, Comprehensive Reviews in Food Science and Food Safety. Volume 3, Issue 2: Published by International Life Sciences Institute, Washington, DC, and Institute of Food Technologists.

numbers of phytochemicals suggest that the potential impact of phytochemicals and functional foods on human and animal health is worth examining as targets of biotechnology efforts.

18.6.1.4 Metabolic Engineering

Developing plants with improved quality traits involves overcoming a variety of technical challenges inherent to metabolic engineering efforts. Both traditional plant breeding and biotechnology techniques are needed to metabolically engineer plants with desired quality traits. Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to improve the production of existing compounds, produce new compounds, or mediate the degradation of undesirable compounds. Significant progress has been made in recent years in the molecular dissection of many plant pathways and in the use of cloned genes to engineer plant metabolism. Specific examples of metabolic engineering of plants to improve nutritional quality at the macro (protein, carbohydrates, lipids, fiber) and the micro level (vitamins, minerals, phytochemicals) and amelioration of antinutrients are discussed in Sections 18.6.2 and 18.6.3.

Although there have been numerous success stories using this approach, there has been an even greater number of studies that have yielded unanticipated results. Trait modifications with the additions of one or two genes produce targeted, predictable outcome. For metabolic pathway manipulations, however, this is not the case. This underscores the fragmented state of our understanding of plant metabolism and highlights the growing gap between our ability to clone, study, and manipulate individual genes and proteins, and our understanding of how they are integrated into and impact the complex metabolic networks in plants. The unexpected outcomes drive home the point that a thorough understanding of the individual kinetic properties of enzymes may not be informative as

to their role. They also make clear that caution must be exercised when extrapolating individual enzyme kinetics to the control of flux in complex metabolic pathways. Regulatory oversight of engineered products has been designed to detect such unexpected outcomes in biotech crops and, as more metabolic modifications are made, new methods of analysis may be needed.

18.6.2 Macronutrients

18.6.2.1 Protein

Most plants have a poor balance of essential amino acids relative to the needs of animals and humans. The cereals (maize, wheat, rice, etc.) tend to be low in lysine, whereas legumes (soybean, peas) are often low in the sulfur-rich amino acids, methionine and cysteine. Successful examples of improving amino acid balance to date include high-lysine maize [65], canola, and soybeans [23]. Consumption of foods made from these crops potentially can help to prevent malnutrition in developing countries, especially among children. Poultry, swine, and other nonruminant animals have specific requirements for each of the essential amino acids. The primary requirements for maize and soybean meal-based diets are lysine in mammals and methionine in avian species. High-lysine and high-methionine maize and soybeans could allow diet formulations that reduce animal nitrogen excretion by providing an improved balance of essential amino acids. When they are out of balance, the amino acid in excess results in increased nitrogen excretion. That balance can be accomplished now, but only by adding costly synthetic lysine and methionine to the diet.

One method of modifying storage protein composition is to introduce heterologous or homologous genes that code for proteins containing elevated levels of sulfur-containing amino acids (methionine, cysteine) and lysine. An 11 kDa synthetic protein, MB1, was created to contain the maximum number of essential amino acids methionine, threonine, lysine, and leucine in a stable, helical conformation, and the structure was also designed to resist proteases to prevent degradation *in planta* [5]. The amino acid composition of MB1, 16% methionine, and 12% lysine, make it a desirable candidate for improving soy protein quality. In transformation vectors, MB1 was targeted to seed protein storage bodies using appropriate leader sequences and seed-specific promoters [79].

An albumin gene for a protein from *Amaranthus* that is nonallergenic (unlike Brazil nut albumin) and rich in all essential amino acids has been introduced into potato [13]. There was a striking increase in the growth rate and production of tubers in transgenic populations compared to the control and also of the total protein content with an increase in most essential amino acids [13]. This study documented, in addition to successful nutritional improvement of potato tubers, the feasibility of genetically modifying other nonseed food crop plants with novel protein composition, such as modification of sweet potato plants with an artificial storage protein (ASP-1) gene [70]. These transgenic plants exhibited a two- and five-fold increase in the total protein content in leaves and roots, respectively, over that of control plants. A significant increase in the level of essential amino acids such as methionine, threonine, tryptophan, isoleucine, and lysine was also observed [70]. A key issue that needs to be kept in mind is that the total amount and composition of storage proteins is not altered to the detriment of the development of the crop plant when attempting to improve amino acid ratios [72].

Attempts to manipulate the lysine content of seeds illustrate that one needs to consider catabolic, as well as anabolic, variables when trying to engineer a particular metabolic phenotype in plants. A key step in lysine synthesis is catalyzed by dihydrodipicolinate synthase, which is feedback inhibited by the pathway end product, lysine, and thus plays a key role in regulating flux through the pathway. Engineering plants to overexpress a feedback insensitive bacterial dihydrodipicolinate synthase greatly increased flux through the lysine biosynthetic pathway. However, in most cases, this did not result in greater steady-state lysine levels because the plants also responded by increasing flux through the lysine catabolic pathway through the elevation of lysine-ketoglutarate reductase. Substantial increases in lysine only occurred in plants where flux increased to such a level that the first enzyme of the catabolic pathway became saturated [8].

18.6.2.2 Carbohydrates

Plants make both polymeric carbohydrates (e.g., starches and fructans) and individual sugars (e.g., sucrose and fructose). The biosynthesis of these compounds is sufficiently understood to allow the bioengineering of their properties, or to engineer crops to produce polysaccharides not normally present.

Genes responsible for the synthesis of fructans can be used to modify plants of higher agronomic value to produce this polymeric carbohydrate. Fructans are an important ingredient in functional foods because evidence suggests that they promote a healthy colon (as a prebiotic agent) and help reduce the incidence of colon cancer. The crop of predominant interest for elevated fructan production is the sugar beet, because the major storage compound of this species is sucrose, the direct precursor for fructan biosynthesis. Sévenier et al. [78] has reported high-level fructan accumulation in a transgenic sugar beet without adverse affects on growth or phenotype. This work has implications both for the commercial manufacture of fructans and also for the use of genetic engineering to obtain new products from existing crops. A similar approach is being used to derive soybean varieties that contain some oligofructan components that selectively increase the population of beneficial species of bacteria (e.g., *Bifidobacteria*) in the intestines of humans and certain animals and inhibit growth of harmful species of bacteria (e.g., *E. coli* 0157:H7, *Salmonella*, etc.) [15]. Thus, high-oligofructan soybeans have the potential to displace some of the antibiotics that are currently used to combat disease caused by pathogenic bacteria. When colonic bacteria ferment dietary fiber or other unabsorbed carbohydrates, the products are short-chain saturated fatty acids. These short-chain fatty acids may enhance absorption of minerals such as iron, calcium, and zinc [90].

The soluble oligosaccharides, stachyose, and raffinose, can cause flatulence and digestive problems [39,85], producing discomfort in humans. The raffinose and stachyose components in conventional soybean or soybean meal used for livestock feed are not digested by nonruminant animals, resulting in reduced feed efficiency. Researchers found that the incorporation of low-stachyose soybean meal in prestarter pig diets tends to improve growth performance [75]. In addition, the increased sucrose content of low-stachyose soyfoods results in a sweeter taste than their traditional counterparts. Manipulating the level of this family of oligosaccharides through rDNA technology has been achieved by inhibiting galactinol synthase activity [51]. This is the first committed step in the stachyose biosynthetic pathway and yields galactinol from UDP-Gal and myo-inositol. The individual members are then synthesized by distinct galactosyl transferases (e.g., raffinose synthase and stachyose synthase). As raffinose and stachyose may be crucial during seed development and storage, perhaps an alternate strategy would be that suggested by Griga et al. [36], which is based on the transfer of α-galactosidase from a thermophilic bacterium (*Thermotoga neapolitana*) into legumes and inducing α-galactosidase to degrade the oligosaccharides after harvesting by thermal potentiation of the α-galactosidase.

Engineering starch content in potatoes is also of interest. Plant ADP glucose pyrophosphorylase (ADPGPP) is sensitive to allosteric effectors and has been proposed to be a key regulator in limiting starch biosynthesis. Stark et al. [83] engineered wild-type and mutant allosterically insensitive *E. coli* ADPGPP for chloroplast-targeted tuber-specific expression in potatoes. Tubers from potato plants transformed with the allosterically insensitive *E. coli* ADPGPP enzyme had starch levels up to 40% higher than the wild type. The higher starch content results in less fat absorption during frying, as moisture lost during frying is replaced by oil uptake. However, there are still problems of irregular granule distribution throughout the tuber.

The amylose:amylopectin ratio has the greatest influence on the physicochemical properties of the starch, and for many applications it is desirable to have a pure or enriched fraction of either amylopectin or amylose. Schwall et al. [77] created a potato producing very high amylose (slowly digested) starch by inhibiting two enzymes that would normally make the amylopectin type of starch that is rapidly digested. This "resistant starch" is not digested in the small intestine, but is fermented in the large intestine by the microflora. Clinical studies have demonstrated that

resistant starch has similar properties to fiber and has potential physiological benefits in humans [15,74].

18.6.2.3 Fiber

Fiber is a group of substances chemically similar to carbohydrates but nonruminant animals poorly metabolize fiber for energy or other nutritional uses. Fiber is only found in foods derived from plants and never occurs in animal products. Fiber provides bulk in the diet such that foods rich in fiber offer satiety without contributing significant calories. Current controversies aside, there is ample scientific evidence to show that prolonged intake of dietary fiber has various positive health benefits, especially the potential for reduced risk of colon and other types of cancer.

Fiber type and quantity are undoubtedly under genetic control, although this topic has been little studied. The technology to manipulate fiber content and type by genetic engineering would be a great benefit to the health status of many individuals who refuse, for taste or other reasons, to include adequate amounts of fiber in their daily diet. For example, fiber content could be added to more preferred foods or the more common sources of dietary fiber could be altered for greater health benefits.

Other fiber-associated compounds include lignans. The two primary lignans of mammalian interest, enterodiol and its oxidation product, enterolactone, are formed in the intestinal tract by bacterial action on plant lignan precursors. Flaxseed is the richest source of mammalian lignan precursors. Because enterodiol and enterolactone are structurally similar to both naturally occurring and synthetic estrogens, and have been shown to possess weakly estrogenic and antiestrogenic activities, they may play a role in the prevention of estrogen-dependent cancers [73]. Genes encoding all the enzymes for the conversion of coniferyl alcohol (lignan and lignin precursor) to secoisolariciresinol, a major dietary phytoestrogen, have been cloned.

However, as discussed in the anti-nutrient section, for animals, low-fiber feedstuffs are often favored. Nonruminant animals do not produce enzymes necessary to digest cellulose-based plant fiber. Plants low in fiber should yield more digestible and metabolizable energy and protein, and less manure and methane when fed to simple stomached species [63]. Improved fiber digestibility in ruminants will have similar beneficial effects as the efficiency of digestion of most high-fiber diets for ruminants is far from optimized.

18.6.2.4 Novel Lipids

Gene technology and plant breeding are combined to provide powerful means for modifying the composition of oilseeds to improve their nutritional value and provide the functional properties required for various food oil applications. Beyond this, research is also being directed at the development of plants that can be used for the production of industrial oils and chemicals that are currently produced from petrochemical sources.

Genetic modification of oilseed crops can provide an abundant, relatively inexpensive source of dietary fatty acids with wide ranging health benefits. Production of such lipids in vegetable oil provides a convenient mechanism to deliver healthier products to consumers without the requirement for significant dietary changes. Major alterations in the proportions of individual fatty acids have been achieved in a range of oilseeds using conventional selection, induced mutation and, more recently, PTGS. Examples of such modified oils include low- and zero-saturated fat soybean and canola oils, canola oil containing medium chain fatty acids (MCFA), high stearic acid canola oil (for *trans* fatty acid-free products), high oleic acid (monounsaturated) soybean oil, and canola oil containing the polyunsaturated fatty acids (PUFA), λ-linolenic acid (GLA; 18:3 n-6), stearidonic acids (SDA; C18:4 n-3), and ω-3 fatty acids [92]. These modified oils are being marketed and many countries have a regulatory system in place for the premarket safety review of novel foods produced through conventional technology.

Medium chain fatty acids range from 6 to 10 carbons long and are only minor components of natural foods with the exception of coconut and palm kernel oils. For example, <5% of the fat in butter is MCFA, and corn oil contains none. Medium chain triglycerides (MCTs) are metabolized differently than long-chain triglycerides (LCTs). MCTs bypass the lymphatic system and MCTs are rapidly oxidized as a quick source of energy. They also aid in absorption of calcium and magnesium [28]. When MCTs are substituted for LCTs in the diet, animals gain less weight, store less adipose tissue, and experience an increase in metabolic rate [2,30]. Mice fed diets with MCT have also been shown to possess increased endurance in swimming tests over that of mice fed diets with LCT [28]. It is probably these factors that have brought MCT oil into the market of ergogenic aids and dietary supplements. Because MCT are not readily available in high quantities in ordinary foods, they must be fed via an artificial formula. This has made them of great interest to researchers. Dehesh et al. [17] has used the mushroom *Morilena* and plants to hunt for enzymes involved in the production of capric and caprylic acid containing MCTs. Expression of an acyl-ACP thioesterase cDNA from *Cuphea hookeriana* in seeds of transgenic canola, an oilseed crop that normally does not accumulate any capric and caprylic acid, resulted in a dramatic increase in the levels of these two MCTs [17].

Edible oils rich in monounsaturated fatty acids provide improved oil stability, flavor, and nutrition for human and animal consumption. Oleic acid (18:1), a monounsaturate, can provide more stability than the polyunsaturates, linoleic (18:2) and linolenic (18:3). From a health aspect, the monounsaturates are also preferred. Antisense inhibition of oleate desaturase expression in soybean resulted in oil that contained >80% oleic acid (23% is normal) and had a significant decrease in PUFA [53]. High-oleic soybean oil is naturally more resistant to degradation by heat and oxidation, and so requires little or no postrefining processing (hydrogenation), depending on the intended vegetable oil application. Lauric acid (C12:0) is known for its good antimicrobial properties, and the monoacylglycerol derivative of lauric acid, monolaurin, is known to have even more potent antimicrobial properties against lipid-coated RNA and DNA viruses, numerous pathogenic Gram positive bacteria, and various pathogenic protozoa. Laurical was the first commercial genetically engineered food oil. Genes from the California bay laurel coding for lauroyl ACP thioesterase were cloned and transferred to canola (low-erucic acid rapeseed) oil crops. The FDA approved Laurical for use in food products [19].

There are many food quality and health considerations that encourage the development of oils containing altered ratios of saturated/unsaturated fatty acids. For example, solid glycerides from highly saturated (high-stearic) oils may have novel functionalities, will contain no *trans* fatty acids, and may be less cholesterolemic than hydrogenated fats. Stearate (stearic acid) is the only saturated fatty acid that has been proven to be neutral with respect to serum cholesterol. One explanation is that after absorption, stearic acid is very quickly converted to oleic acid, a monounsaturated fatty acid. Because stearate is solid at room temperature, high-stearate soybean oil and canola (achieved through antisense inhibition of stearoyl-ACP desaturase) will require little or no postrefining processing such as chemical hydrogenation to saturate bonds in order to produce a solid spread at room temperature.

A great deal of research is currently aimed at evaluating preliminary evidence that suggests conjugated linoleic acid (CLA), a fatty acid found in lipids of ruminants, is a naturally occurring anticarcinogen. The potential benefits of this unique fatty acid include anticarcinogenic, antiatherogenic, and antidiabetic properties and altered immune response. Findings provide evidence for a link between CLA, eicosanoids, the control of lipid accumulation in adipocytes, and effects of CLA on the immune system [15].

A key function of α-linolenic acid (ALA) is as a substrate for the synthesis of longer-chain ω-3 fatty acid found in fish, eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3), which are present in the retina of the eye and cerebral cortex of the brain. DHA is also the predominant structural fatty acid in the gray matter of the brain. It is believed that EPA and DHA play an important role in the regulation of inflammatory immune reactions and blood pressure, brain development *in utero*, and, in early postnatal life, the development of cognitive function. SDA, EPA, and DHA also possess anticancer properties. Research indicates that the ratio of n-3 to n-6 fatty acids

may be as important to health and nutrition as the absolute amounts present in the diet or in body tissues. Current Western diets tend to be relatively high in n-6 fatty acids and relatively low in n-3 fatty acids. This is due to our high intake of vegetable oils, which are rich in n-6 fatty acids, and our low intake of oils and foods rich in n-3 fatty acids, such as fatty fish. Production of a readily available source of long-chain-PUFA, specifically ω-3 fatty acids, delivered in widely consumed prepared foods could deliver much needed ω-3-fatty acids to large sectors of the population with skewed n-6:n-3 ratios. In plants, the microsomal ω-6 desaturase-catalyzed pathway is the primary route of production of polyunsaturated lipids. Ursin [89] has introduced the Δ-6 desaturase gene from a fungus (*Mortierella*) succeeding in producing ω-3 fatty acids in canola. In a clinical study designed to determine the relative efficacy of various fatty acids, metabolism of ALA and SDA to EPA, DPA (n-3 docosapentaenoic acid), and DHA in humans was measured. James et al. [45] observed that SDA was superior to ALA as a precursor by a factor of 3.6. Transgenic canola oil was obtained that contains >23% SDA, with an overall n-6:n-3 ratio of 0.5. Oil with this ratio could help to redress the off-balance Western diet of high n-6:n-3. A similar approach has been used to produce healthy fat livestock as outlined in the animal section.

Many plant lines will be engineered to produce diverse oils having well-defined uses in the food, detergent, and lubricants industries. Now through biotechnology, specialty oils may also be developed with pharmaceutical and chemical feedstock applications in mind. The genetic engineering of oilseed plants promises to have broad impact on the development of new crops for both new and existing applications.

18.6.3 Micronutrients

18.6.3.1 Vitamins and Minerals

The clinical and epidemiological evidence is clear that select minerals (iron, calcium, selenium, and iodine) and a limited number of vitamins (folate, vitamins E, B_6, and A) play a significant role in maintenance of optimal health and are limiting in diets worldwide.

Vitamin E levels are being increased in several crops, including soybean, maize, and canola, while rice varieties are being developed with the enhanced vitamin A precursor, β-carotene, to address vitamin A deficiency that leads to tragic consequences in many developing countries. Other targets include improved iron content, through the production of iron-rich storage protein, bioavailable phosphorus released from phytate, and isoflavonoids.

As with macronutrients, one way to ensure an adequate dietary intake of nutritionally beneficial phytochemicals is to adjust their levels in plant foods. Until recently, such work had been hindered by the difficulty in isolating the relevant genes (e.g., for vitamin biosynthesis). However, the advent of genomics during the past few years has provided new routes for such work. Using nutritional genomics, DellaPenna [18] isolated a gene, γ-tocopherol methyltransferase (γ-TMT), which converts the lower activity precursors to the highest activity vitamin E compound, α-tocopherol. With this technology, the vitamin E content of *Arabidopsis* seed oil has been increased nearly ten-fold and progress has been made to move the technology to agricultural crops such as soybean, maize, and canola. Vitamin A is an essential micronutrient and widespread dietary deficiency of this vitamin in rice-eating Asian countries has tragic consequences. Five million children in South East Asia develop an eye disease called xerophthalmia every year, and 250,000 of them eventually become blind [15]. Improved vitamin A nutrition would alleviate this serious health problem and, according to UNICEF, could also prevent up to 2 million infant deaths because vitamin A deficiency predisposes them to diseases such as measles.

Rice is a staple that feeds nearly half of the world's population, but milled rice does not contain any β-carotene or its carotenoid precursors. Integrating observations from prokaryotic systems into their work enabled researchers to clone the majority of carotenoid biosynthetic enzymes from plant during the 1990s. A research team discovered that immature rice endosperm is capable of synthesizing the early intermediate of β-carotene biosynthesis [93]. Using carotenoid pathway genes from daffodil

and *Erwinia* and a Rubisco (CO_2-fixing enzyme) transit peptide, this team succeeded in producing β-carotene in the rice endosperm. This major breakthrough lead to the development of "golden rice" and shows that an important step in provitamin A synthesis can be engineered in a nongreen plant part that normally does not contain carotenoid pigments. A similar method was used by Monsanto to produce β-carotene in canola.

Iron is the most commonly deficient micronutrient in the human diet, and iron deficiency affects an estimated 1–2 billion people. Anemia, characterized by low hemoglobin, is the most widely recognized symptom of iron deficiency, but there are other serious problems such as impaired learning ability in children, increased susceptibility to infection, and reduced work capacity. Two research groups [35,69] employed the gene for ferritin, an iron-rich soybean storage protein, under the control of an endosperm-specific promoter. Grain from transgenic rice plants contained three times more iron than normal rice. To increase the iron content in the grain further, the researchers focused on iron transport within the plant [54]. Table 18.4 presents examples of crops that have already been genetically modified with macro- and micronutrient output traits that may provide health benefits to consumers and domestic animals [15].

18.6.3.2 Phytochemicals

The search for new compounds to prevent and/or treat human disease has led to the formation of specialized biotechnology firms searching for nutraceuticals. To obtain such therapeutic levels in the diet, additional fortification of the food supply will be required as well as modification of dietary preferences or direct modification of micronutrient levels in food crops. However, studies by Bacon et al. [3] demonstrate that maximized dietary intake is not always correlated with optimized dietary benefit. Quercetin is a flavonoid that has been demonstrated to work optimally at low concentrations in protecting against cancerous cell proliferation and the actions of the carcinogen PhIP found in cooked meat; however, as the concentration increased the effect was ameliorated. After the carcinogen PhIP is activated in the liver, PhIP can attack DNA to form DNA adducts. Quercetin can inhibit DNA adduct formation in a dose-dependent manner. It is to be expected that similar effects may be found for other flavonoids/phytochemicals once effective experiments designed to evaluate systems are developed. This, of course, illustrates the importance of taking a cautious approach to any research to increase phytochemicals with putative beneficial effects under the premise of "more is better."

Unlike vitamins and minerals, the primary evidence for the health-promoting roles of phytochemicals comes from epidemiological studies, and the exact chemical identity of many active compounds has yet to be determined. However, for select groups of phytochemicals, such as nonprovitamin A carotenoids, glucosinolates, and phytoestrogens, the active compound or compounds have been identified and rigorously studied. A great irony of nature is that the body's natural metabolism involving oxygen also produces a host of toxic compounds called "free radicals." These compounds can harm body cells by altering molecules of protein and fat and by damaging DNA. Antioxidants counteract, or neutralize, the harmful effects of free radicals.

Epidemiological studies suggest that Brassica vegetables are protective against cancers of the lungs and alimentary tract. Cruciferous vegetables are the dietary source of glucosinolates, a large group of sulfur-containing glucosides. These compounds remain intact unless brought into contact with the enzyme myrosinase by pests, food processing, or chewing. Myrosinase releases glucose and breakdown products, including isothiocyanates. These highly reactive compounds are potent inducers of Phase II (or protective) enzymes *in vitro* (see Chapter 12). Isothiocyanates also inhibit mitosis and stimulate apoptosis in human tumor cells, *in vitro* and *in vivo* [47]. Indole-3 carbinol (I3C) is currently under investigation for its cancer chemo-preventive properties, particularly of the mammary gland and the increased formation of 2-hydroxylated estrogen metabolites (catechol) may protect against cancer [15]. The salicylate hydroxylase (*nahG*) gene from bacteria has been introduced into *Arabidopsis* and tobacco [27] where it successfully converts salicylic acid to catechol. This provides a potential mechanism for modification in edible plants where there is a salicylic precursor.

TABLE 18.4
Examples of Crops Genetically Modified with Nutritionally Improved Traits Intended to Provide Health Benefits to Consumers and Domestic Animals

Crop/Species	Trait	Transgene
Alfalfa	+Phytase	Phytase (*Aspergillus*)
	+Resveratrol	Resveratrol synthase
	Lignin↑	Down-regulation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase
Arabidopsis and tobacco	+Catechol	Salicylate hydroxylase (*nahG*)
Beet	+Fructans	1-Sucrose:sucrose fructosyl transferase
Canola	Vitamin E↑	γ-Tocopherol methyl transferase (*Arabidopsis*)
	Lauric acid↑	Lauroyl ACP thioesterase (California bay tree)
	γ-Linolenic acid↑	Δ-6- and Δ-12 desaturases
	+n-3 Fatty acid	Δ-6 Desaturase gene *(Mortierella)*
	+β-Carotene	Phytoene synthase (daffodil)
		Phytoene desaturase *(Erwinia)*
		Lycopene cyclase (daffodil)
	8:0 and 10:0 Fatty acids MCFA↑	Ch FatB2, a thioesterase cDNA (*Cuphea hookeriana*)
Cassava	Cynaogenic glycosides↓	Hydroxynitril lyase
Cotton	Oleic acid↑	Mutant Δ-12 desaturase
	High-oleic and high-stearic cottonseed oils	hpRNA-mediated PTGS desaturases
Coffee	Caffeine↓	Antisense xanthosine-*N*-7-methyl transferase (coffee)
Lupin	Methionine↑	Seed albumin (sunflower)
Maize	Methionine↑	mRNA stability by intron switching Dzr1 target
	Fumonisin↓	de-esterase + de-aminase (μbial)
	Insect resistance	Avidin (chicken)
	Protein with favorable amino acid profile↑	α-Lactalbumin (porcine)
	Sulfur amino acids↑	Maize 15 kDa-zein
Maize	Vitamin C↑	Wheat dehydroascorbate reductase (DHAR)
Potato	Starch↑	ADP glucose pyrophosphorylase (*E. coli*)
	Very-high-amylose starch↑	Inhibition of SBE A and B
	Inulin molecules↑	1-SST (sucrose:sucrose 1-fructosyltransferase) and the 1-FFT (fructan:fructan 1-fructosyltransferase) genes of globe artichoke (*Cynara scolymus*)
	+Sulfur-rich protein	Nonallergenic seed albumin gene (*Amaranthus hypochondriacus*)
Potato	Solanine↓	Antisense sterol glyco transferase (Sgt) gene
Rice	+β-Carotene	Phytoene synthase (daffodil)
		Phytoene desaturase *(Erwinia)*
		Lycopene cyclase (daffodil)
	↑Iron	Ferritin (*Phaseolus*)
	Iron↑	Ferritin (*Phaseolus*)
		Metallothionein (rice)
		Phytase (mutant, *Aspergillus*)
	Allergenic protein↓	Antisense 16 kDa allergen (rice)

TABLE 18.4 (Continued)

Crop/Species	Trait	Transgene
Rice	+Puroindolinone compounds: softer rice kernels, flour yields more finer particles, less damage to starch	Wheat puroindoline genes
Sorghum	Improved digestibility of livestock feed	Mutated Brown midrib (Bmr) encodes caffeic acid *O*-methyltransferase (COMT), a lignin-producing enzyme
Soybeans	Improved amino acid composition	Synthetic proteins
	Increased sulfur amino acids	Overexpressing the maize 15 kDa zein protein
	Oleic acid↑	Δ-12 Desaturase (soybean, sense suppression)
	Oleic acid↑	Ribozyme termination of RNA transcripts down-regulate seed fatty acid
	Immunodominant allergen↓	Gene silencing of cysteine protease P34 (34 kDa)
Soybean/*arabidopsis*	Isoflavones↑/+isoflavones	Isoflavone synthase
Sweet potato	Protein content↑	Artificial storage protein (ASP-1) gene
Tomato	Provitamin A↑ and lycopene↑	Lycopene *cyclase* (*Arabidopsis*)
	Provitamin A↑	Phytoene desaturase (*Erwinia*)
	Flavonoids↑	Chalcone isomerase (*Petunia)*
	Lycopene↑	Engineered polyamine accumulation
Wheat	Glutenins↑	High molecular weight subunit genes
	Caffeic and ferulic acids↑	Wheat gene

Source: Modified from Chassy, B., *et al.* 2004. Safety and Nutritional Assessments of Foods and Feeds Derived Through Biotechnology, Comprehensive Reviews in Food Science and Food Safety. Volume 3, Issue 2: Published by International Life Sciences Institute, Washington, DC, and Institute of Food Technologists.

In addition to the antioxidant characteristics of vitamin A, other interesting products in the carotenoid pathway include lycopene, which may benefit the cardiovascular system by reducing the amount of oxidized low-density lipoprotein (LDL). Recent epidemiological studies have suggested a potential benefit of this carotenoid in reducing the risk of prostate cancer, particularly the more lethal forms of this cancer. Five studies support a 30–40% reduction in risk associated with high tomato or lycopene consumption in the processed form in conjunction with lipid consumption, although other studies with raw tomatoes were not conclusive [33]. In an intriguing paper that demonstrates the importance of serendipity in scientific investigation, Mehta et al. [58] used a transgenic approach to modify polyamines in tomato fruit to retard the ripening process. These transgenic tomatoes had longer vine lives, suggesting that polyamines have a function in delaying the ripening process. An intriguing corollary to the paper is the unanticipated enrichment in lycopene content of the genetically modified tomato fruit. The lycopene levels increased 2–3.5 times compared to the nonengineered tomatoes. This is a substantial enrichment, exceeding that so far achieved by conventional means. This novel approach should work in other fruits and vegetables.

Isoflavones have drawn much attention because of their potential benefits to human health. The isoflavones genistein and daidzein are naturally occurring plant compounds that are being studied for their substantial health benefits. They are found almost exclusively in soybeans and other leguminous plants. They serve as the signal molecule for establishing the symbiotic relationship between plants and rhizobial bacteria resulting in the formation of nitrogen-fixing root nodules. The reported health

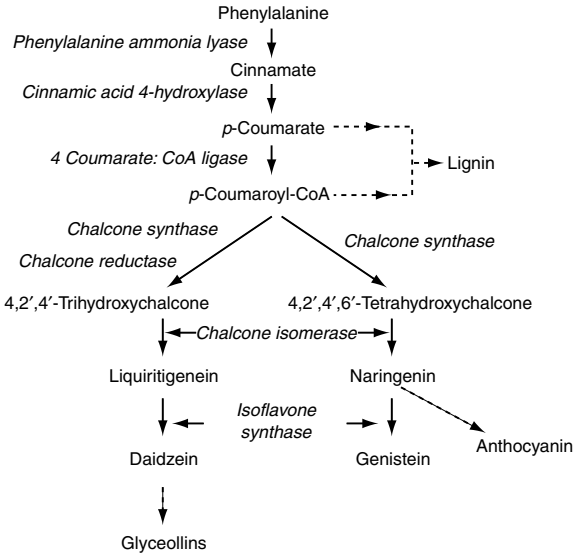


FIGURE 18.15 A simplified diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis.

benefits include estrogenic and anticancer activity, assistance in the prevention of artherogenic oxidation of low-density lipoproteins, and increasing bone mass. The basis for these effects has not been established, but the weak estrogenic activity of isoflavones, which are sometimes referred to as phytoestrogens, may be a factor in conferring these properties. There is much varietal and regional variability in quantity and losses of isoflavones due to processing of seeds for traditional soy foods or protein products. Increasing isoflavone concentrations and reducing their variability in soybean seeds could address the problem of achieving efficacious levels. Isoflavones are synthesized as part of the phenylpropanoid pathway (Figure 18.15) [49]. This pathway has multiple branches that lead to numerous secondary metabolites including lignins, flavanols, anthocyanins, and some phytoalexins. The key enzyme that initiates isoflavonoid synthesis is a legume-specific isoflavone synthase (IFS).

Modification of the IFS gene should allow manipulation of the phenylpropanoid pathway for both agronomic and nutritional purposes. Expected in 2007 are soybeans derived via biotechnology that will contain approximately four times the amount of isoflavones as wild-type soybeans.

Stilbenes, including resveratrol, are phenolic phytochemicals that accumulate in a wide range of plant species, including pine, grapevine, peanut, and rhubarb. Grape-derived foods such as grapes, raisins, and red wine are among the few human dietary sources of resveratrol. Resveratrol has attracted considerable notice as a compound with possible beneficial effects on human health. Resveratrol is an excellent antioxidant, inhibiting platelet aggregation, and is thought to contribute to improved heart function based on epidemiological studies. It was shown to have "chemo-preventive" activity, preventing the formation of tumors in mouse skin bioassays, and, therefore, may help reduce cancer rates in humans [46]. Hipskind and Paiva [43] have genetically engineered the constitutive accumulation of a resveratrol glucoside in alfalfa leaves and stems.

Other phytochemicals of interest are listed in Table 18.3. Although there is a growing knowledge base indicating that elevated intakes of specific phytochemicals may reduce the risk of diseases, such as certain cancers, cardiovascular diseases, and chronic degenerative diseases associated with aging, further research is still required to prove definitive relationships.

18.6.3.3 Antinutrients

Plants produce many phytochemicals and other defense strategies to protect themselves from predators. For example, phytate, a plant phosphate storage compound, is an antinutrient because

it strongly chelates iron, calcium, zinc, and other divalent mineral ions, making them unavailable for uptake. Nonruminant animals generally lack the phytase enzyme needed for digestion of phytate. Poultry and swine producers in most countries currently add mined and processed (powdered) phosphate to their feed rations to enable optimal growth. Excess phosphate is excreted into the environment resulting in water pollution. When low-phytate soybean meal is utilized along with low-phytate maize for animal feeds, phosphate excretion in swine and poultry manure is halved. Potrykus [69] has developed a series of transgenic rice lines to address this problem. One approach has been to reduce the phytate in rice endosperm by introducing a gene that encodes for a heat-stable phytase from *Aspergillus fumigatus*. This enzyme can survive boiling and has two pH optima—acidic to match the stomach and alkaline to match the intestine. To promote the reabsorption of iron, a gene for a metallothionein-like protein has also been engineered. Low-phytate maize was commercialized in the United States in 1999 [91]. Research indicates that the protein in low-phytate soybeans is also slightly more digestible than the protein in traditional soybeans. Austin-Phillips et al. [1] have genetically engineered alfalfa to produce phytase. In a poultry feeding trial, better results were obtained using transgenic plant material than with the commercially produced phytase supplement. Several studies have shown that optimal performance and bone mineralization can result from diets without added phosphorus when phytase is included [52]. Poultry grew well on the engineered alfalfa diet without any inorganic phosphorus supplement, which shows that plants can be tailored to increase the bioavailability of this essential mineral. Thus, phosphorus supplements may be eliminated from poultry feed, to reduce costs and pollution.

Other antinutrients that are being examined as possible targets for reduction are trypsin inhibitors, lectins, and several other heat-stable components found in soybeans. Consideration must be given to possible increased susceptibility to pests and diseases when natural toxicants are removed, so the base germplasm should have input traits to counter this. Reducing the amounts of trypsin inhibitors in soybeans would have a positive effect on the domestic feed industry and offer a competitive advantage for on-farm feeding of this protein source. If this can be combined with increases in the amounts of essential amino acids, very large improvements in productivity may be achieved.

18.6.3.4 Allergens

Although symptoms of food intolerance are common, true food allergy is less common [86]. A food allergy is distinguished from food intolerance and other disorders by the production of antibodies (IgE) and the release of histamine and similar substances. The immune system responds to ingestion of a particular food or food component. The symptoms may be localized to the stomach and intestines or may involve many parts of the body after the food is digested or absorbed. The symptoms usually begin immediately, and are almost always evident within a couple of hours after eating. The best characterized true allergens include the superfamily cupins, which include globulins found in nuts and beans and albumins in nuts, the superfamily prolamins, which are found in cereals and others such as heverin, which causes contact dermatitis from latex and chitinases [86].

Foods that frequently cause malabsorption or other food intolerance syndromes other than direct immune responses include wheat and other gluten-containing grains (celiac disease or gluten-sensitive enteropathy is a multifactorial disorder caused by an inappropriate T-cell-mediated response to ingested gluten, resulting in chronic intestinal inflammation characterized by villous atrophy and malabsorption [15]). Also, cow's milk (milk/lactose intolerance and intolerance of dairy products) and maize products evoke common food allergies. According to present evidence [10], thioredoxin may be used to improve foods through, among other changes, lowering allergenicity and increasing digestibility. Thioredoxin reduces disulfide bonds of allergens (convert S—S to 2 SH) and thereby alters the allergenic properties of disulfide proteins extracted from wheat flour. By changing the levels of expression of the thioredoxin gene scientists have been able to reduce the allergenic effects of wheat and other cereals using a canine model system.

The list of antinutritional components found in soybean meal and soyfoods includes the trypsin inhibitors, lectins, and several heat-stable components. The trypsin inhibitors in soybeans are the

major antinutritional components of this protein source. Because trypsin inhibitor is a sulfur-rich protein, lines without or with low amounts of trypsin inhibitor will be slightly deficient in essential amino acids, particularly the sulfur-containing amino acids. This can be countered by introducing the Mb1 protein (discussed earlier).

One soy storage protein accounts for 85% of IgE responses in soy-sensitive individuals. Gly m Bd 30K, a member of the papain superfamily of cysteine proteases also referred to as P34 (34 kDa), has been identified as a major allergen in soybean seeds. Gene silencing, driven by a seed-specific β-conglycinin promoter, was used to eliminate the accumulation of P34 in transgenic soybeans, removing the principal source of food allergenicity in soy. Early results from human blood serum tests indicate that P34-specific IgE antibodies could not be detected after consumption of gene-silenced beans [40,41]. Reduced allergens and trypsin inhibitors and increased limiting amino acids levels in soybeans would have a positive effect on the domestic feed industry and offer a competitive advantage for on-farm feeding of this protein source.

18.6.3.5 Toxins

Plants are not always benign and produce many phytochemicals to protect themselves from pests. Over years of breeding and selection, most of the genes involved in the production of noxious products have been eliminated from plants used as food and feed crops. Potatoes and tomatoes are members of the nightshade family (Solanaceae) and can contain toxic glycoalkaloids (e.g., solanine) that have been linked to spina bifida [26]. A number of people die each year from cyanogenic glycosides from cassava [37]. Biotechnology approaches can be employed to downregulate or even eliminate the genes involved in the metabolic pathways for the production, accumulation, and/or activation of these toxins in plants. For example, the solanine content of potato has already been reduced substantially using an antisense approach, and efforts are underway to reduce the level of other major potato glycoalkaloid, chaconine [57]. Work has also been done to reduce cyanogenic glycosides in cassava through expression of the cassava enzyme hydroxynitrile lyase in the roots [80].

18.6.4 The Future of Crop Biotechnology

For essential minerals and vitamins that are limiting in world diets, the need and way forward is clear, and improvement strategies should be pursued, as long as attention is paid to the upper safe limit of intake for each nutrient. However, for many other health-promoting phytochemicals, clear links with health benefits remain to be demonstrated. Such links, if established, will make it possible to identify the precise compound or compounds to target and which crops to modify to achieve the greatest nutritional impact and health benefits. Because these decisions will require an understanding of plant biochemistry, human and animal physiology, and food chemistry, strong interdisciplinary collaborations will be needed among plant scientists, nutritionists, and food scientists to ensure a safe and healthful food supply.

18.7 ANIMALS

The development of the capacity to modify animals at the molecular level has expanded their roles especially in the areas of evaluating therapies for specific diseases, and "molecular pharming," including the production of valuable products in milk. Transgenic animals have tremendous potential to act as valuable research tools in the agricultural and biological sciences. They can be modified specifically to address scientific questions that were previously difficult if not impossible to determine.

Notwithstanding the advent of successful nuclear transfer technology with the dawn of Dolly (the first cloned sheep), the most widely used technique for the production of transgenic animals is by microinjection of DNA into the pronucleus of a recently fertilized egg (Figure 18.16). Using various

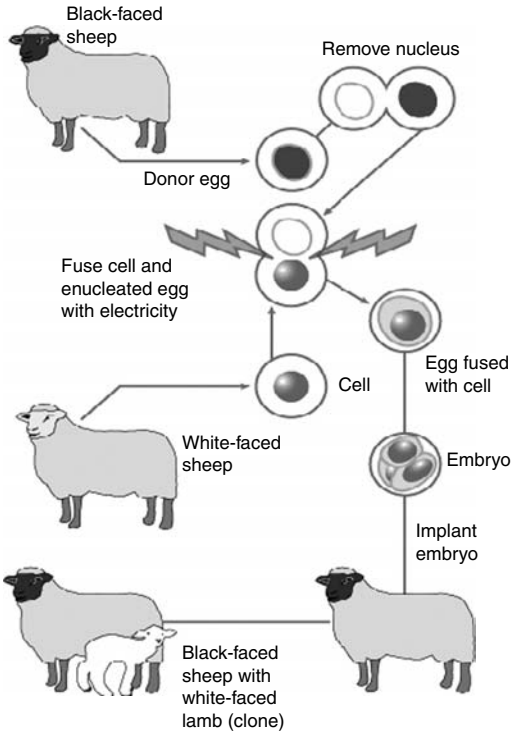


FIGURE 18.16 Dolly and mom. (Courtesy of Ian Wilmut.)

transgenic tools such as antisense technology (putting a reverse copy to switch off expression), it is now possible to add a new gene to the genome, increase the level of expression or change the tissue specificity of expression of a gene, or decrease the level of synthesis of a specific protein [81]. An additional factor added by the new nuclear transfer technology is the capability of removing or altering an existing gene via homologous recombination.

18.7.1 Methods for Producing Transgenic Animals

The steps involved in the production of transgenic animals are as follows:

- Integration of retroviral vectors into an early embryo.
- Retroviral infection of germinal vesicles oocytes.
- Injection of DNA into the pronucleus of a newly fertilized egg.
- The incorporation of genetically manipulated embryonic stem (ES) cells into an early embryo.
- The incorporation of genetically manipulated primordial germ cells into an early embryo.
- Sperm delivery.
- Nuclear transplantation.
- Microprojectile injection.

Transgenic technology has been applied to several animals including agricultural species such as sheep, cattle, goats, pigs, poultry, and fish. The applications for transgenic animal research fall broadly into two distinct areas, namely, medical and agricultural applications. The recent focus on developing animals as bioreactors to produce valuable proteins in their milk can be catalogued under both areas. Underlying each of these, of course, is a more fundamental application, that is, the use

of those techniques as tools to ascertain the molecular and physiological bases of gene expression and animal development. This understanding can then lead to the creation of techniques to modify development pathways.

As noted by Pinkert and Murray [66], there are still fundamental limitations to the widespread use of transgenic technology in all animals except the mouse. Limitations include (1) lack of knowledge concerning the genetic basis of factors limiting production traits; (2) identification of tissue and developmentally specific regulatory sequences for use in developing gene constructs, expression vectors, and in gene targeting; and (3) establishment of novel methods to increase efficiency of transgenic animal production.

In medicine the most versatile animal model has been the mouse and the technology has been perfected to customize the engineering of mice. Until recently, the principle advantage that the mouse held over all other species was the ability to isolate with relative ease cells that remain amenable to engineering called ES cells. ES cells are derived from the inner cell mass of the blastocyst formed during early embryogenesis. Distinguished from all other stem cells, they are pluripotent, able to develop into virtually any and all cells and tissues in the body; and, consistent with their expression of telomerase, self-renewing, a potentially limitless source of cells. One of the areas in which the mouse has been supreme is the ability, using these ES cells, to target with great specificity regions within chromosomes via what is termed homologous recombination. Using this method, researchers can (1) incorporate a novel foreign gene into a genome, for example, to improve the lipid profile in milk; (2) modify an endogenous gene; or (3) delete a portion of a specific endogenous gene creating a "loss-of-function" mutant termed a "knock-out" to study phenotypic effects of inactivating genes.

Until 1998, isolating ES cells in other mammals proved elusive, but in a milestone paper [87] James Thomson at University of Wisconsin-Madison reported the first successful isolation, derivation, and maintenance of a culture of human ES cells (hES cells). Obviously, this is not the focus of this chapter but it is interesting to note this leap made from mouse to man. To paraphrase Thomson, these cells are different from all other human stem cells isolated to date and as the source of all cell types, they hold great promise for use in transplantation medicine, drug discovery and development, and the study of human developmental biology.

18.7.2 Agricultural Applications

Since 1985, transgenic farm animals harboring growth-related gene constructs have been created, although ideal growth phenotypes were not achieved because of an inability to coordinately regulate either gene expression or the ensuing cascade of endocrine events. Using DNA microinjection, the types of genes and regulatory sequences introduced into livestock species become important considerations. To date, for agricultural species, the types of transgenes used fall into two main types, those encoding growth factors and those encoding proteins for expression in the mammary gland. Uses of transgenic animals in agriculture from a food context include:

- To improve meat and milk quality.
- To improve processing characteristics such as for cheese production.
- To produce valuable proteins in milk, blood, or urine.
- Preserve rare or threatened animals.
- To cross species barriers.

Introduction of growth factors alters the efficiency of meat production and alter the partitioning of nutrient resources toward increased lean production. Gene constructs designed to express directly or indirectly various growth factors, and thus to alter body composition, constitute the largest class of transgenes transferred into livestock species. The majority of these transgenes expressed growth hormone (GH), although other constructs based on GH-releasing hormone and insulin-like growth factor-I (IGF-1) also have been used. In general, pigs and sheep expressing these constructs

were leaner and more feed-efficient, but they also suffered from a number of complications as a consequence of the high, unregulated levels of circulating GH, indicating the need for tight control of hormone secretion. In the late 1990s, preliminary trials were conducted on the development of GH and IGF-1 transgenic pigs with enhanced growth performance traits [71]. Preliminary results indicated desirable effects on growth and body composition traits without apparent abnormalities, suggesting that useful animals could become available to swine breeders in the future.

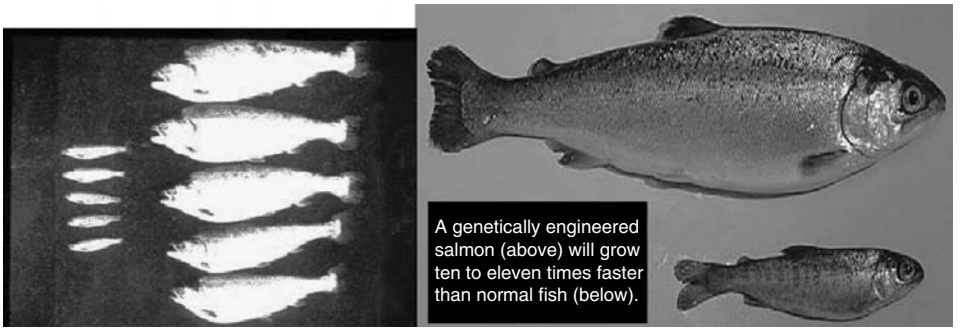
Milk is one of the principle targets for engineering productivity traits. This includes altering milk fat composition, that is, to decrease saturated fatty acids and to increase fats with beneficial functionality. As noted in the plant section, excessive amounts of n-6 PUFA and a very high n-6/n-3 ratio promotes the pathogenesis of many modern diseases (e.g., heart disease, cancer, etc.), while balancing or reducing the ratio of n-6/n-3 fatty acids may decrease the risk of these diseases. Thus, for good health it is necessary to have a balance of n-6/n-3 fatty acids in the diet and in our bodies. Mammals cannot naturally produce ω-3 (n-3) fatty acids from the more abundant ω-6 (n-6) fatty acids and so they must rely on a dietary supply. Kang et al. [50] showed that mice engineered to carry a fat-1 gene from *C. elegans* can add a double bond into an unsaturated fatty acid hydrocarbon chain and convert n-6 to n-3 fatty acids. This results in an abundance of n-3 and a reduction in n-6 fatty acids in the organs and tissues of these mice, in the absence of dietary n-3. As well as presenting an opportunity to investigate the roles played by n-3 fatty acids in the body, their discovery indicates that this technology might be adapted to enrich n-3 fatty acids in animal products such as meat, milk, and eggs.

Current research in food-related animal biotech is focused on the potential uses of transgenic technology in the dairy industry. A reasonable expectation is that genetically engineered dairy cows could become available to the industry within the next two decades. Such genetically engineered animals would be of two types: those with added or altered proteins to increase the yield of cheese and the range of products that can be made from milk, and those that have had the milk altered to yield more healthy milk for human consumption. In the former case, the increased expression of κ-casein and/or α_{s1}-casein, or decreased expression of α-lactoglobulin, could yield animals producing milk specifically for manufacturing purposes. In the latter, an improved lipid profile has already been achieved in goats by James Murray at University of California Davis [55].

18.7.3 Fish

In contrast to poultry studies, work with fish has moved ahead with far greater speed. Gene transfer techniques have been applied to a large number of aquatic organisms, both vertebrates and invertebrates. Gene transfer experiments have targeted a wide variety of applications, including the study of gene structure and function, aquaculture production, and use in fisheries management programs. Because fish have high fecundity, large eggs, and do not require reimplantation of embryos, transgenic fish are attractive models for studying gene expression. Transgenic zebrafish have found utility in studies of embryogenesis, with expression of transgenes marking cell lineages or providing the basis for study of promoter or structural gene function. Although not as widely used as zebrafish, transgenic medaka and goldfish have been used for studies of promoter function. This body of research indicates that transgenic fish provide useful models of gene expression, reliably modeling that in "higher" vertebrates.

Perhaps the largest number of gene transfer experiments address the goal of genetic improvement for aquaculture production purposes. The principal area of research has focused on growth performance, and initial transgenic GH fish models have demonstrated accelerated and beneficial phenotypes. DNA microinjection methods have propelled the many studies reported and have been most effective due to the relative ease of working with fish embryos. Bob Devlin's group at the University of British Columbia, Vancouver [20] has demonstrated extraordinary growth rate in coho salmon which were transformed with a GH from sockeye salmon (Figure 18.17). The transgenic fish achieve up to 11 times the size of their wild-type littermates within 6 months, reaching maturity in about half the time. Contrary to some reports, these fish do not grow to abnormal size; they merely



Fish attractive candidates

- Produce eggs in large quantities outside the body.
- Aquaculture one of the fastest growing food-sectors.
- Since 1984, aquaculture has expanded 10% pa, 3% for livestock meat.
- 1.6 percent rate of growth for capture fisheries (FAO 2000).
- U.S. alone, sale of aquaculture products has grown from $45 million in 1974 to over $978 million in 1998 (National Agricultural Statistics Service 2000).

FIGURE 18.17 Transgenic Salmon. (Courtesy of Bob Devlin.)

reach mature size in less than half the time period of their siblings. Interestingly, this dramatic effect is only observed in feeding pens where the transgenics' ferocious appetites demands constant feeding. If the fish are left to their own devices and must forage for themselves, they appear to be out-competed by their smarter wild-type siblings.

However, most studies, such as those involving transgenic Atlantic salmon and channel catfish, report growth rate enhancement on the order of 30–60%. In addition to the species mentioned, GH genes have also been transferred into striped bass, tilapia, rainbow trout, gilthead sea bream, common carp, bluntnose bream, loach, and other fishes.

The second general area of interest has been the development of lines of transgenic domestic animals for use as bioreactors. One of the main targets of these so-called "gene pharming" efforts has involved attempts to direct expression of transgenes encoding biologically active human proteins. In such a strategy, the goal is to recover large quantities of functional proteins that have therapeutic value, from serum, urine, or from the milk of lactating females. To date, expression of foreign genes encoding lysozyme, alpha-1-antitrypsin (AAT), tissue plasminogen activator, clotting factor IX, protein C, and spider dragline silk were successfully targeted to the mammary glands of goats, sheep, cattle, and/or swine. However as pharmaceutical production is beyond the scope of this chapter these will not be reviewed here.

Animal biotechnology, particularly transgenic animal technology, has been widely researched and practiced in the past decade. Unlike for food production, transgenic farm animals as bioreactors to make human recombinant proteins in their milk or as organ donors for xenotransplantation in humans has been heavily researched and invested in the past decade. However, the current efficiency for producing transgenic animals, particularly farm animals for food and meat production, is low and the cost is high. Success in the production of transgenic farm animals requires an adequate animal facility and dedicated teams of embryologists, veterinarians, animal scientists, and molecular biologists. Improvement on the success rate of the transgenic technology largely relies on the application of (1) various classical reproductive and embryological technologies such as artificial insemination, superovulation, embryo transfer, and so forth and (2) newly emerging contemporary technologies such as cloning and other assisted reproductive technologies.

18.8 DIAGNOSTICS

The growing concern over food safety is having a positive impact on biotechnology. Biotechnology-based diagnostic systems offer more rapid and lower cost alternatives to slower and more costly traditional methods. Given that the large majority of food samples yield negative results when tested for contaminants, it is more cost effective to utilize these diagnostics tests for initial on-site monitoring, and use the more conventional methods for the smaller number of positives and controls.

Food- and water-borne illnesses caused by microorganisms pathogenic to humans have been estimated to affect more than 80 million Americans and cost the U.S. economy over $40 billion annually. For example, some 9000 Americans die each year from food-borne illnesses caused by microorganisms such as *E. coli* in meat and *Salmonella* in poultry. At present, virtually all food inspection is visual. Rapid, accurate, noninvasive tools of biotechnology can help improve the detection and control of food-borne human pathogens as well as chemical contaminants. DNA probe kits have been developed for *Salmonella*, *Listeria*, *E. coli* 0157:H7, and *Staphylococcus aureus*. Compared to traditional culture-plating methods, these new diagnostic kits offer greater precision, shorter turnaround times, and reduced need for highly trained personnel. DNA diagnostic techniques for Norwalk viruses are also commercially available. Culture techniques have not been successful in detecting these leading causes of gastroenteritis, making the availability of genetically based techniques critical to the detection and identification of the Norwalk viruses.

In addition to detecting food contaminants, DNA probes and other tools of biotechnology can help reduce levels of naturally occurring toxicants in foods. DNA probes can be exploited in research and plant breeding to isolate genes associated with the biosynthesis of major toxicants, facilitate understanding of the genetic regulatory mechanisms for toxicants in plants, and develop lines of plants with reduced levels of toxicants.

Mycotoxins (as discussed in the Section 18.6) in food are a periodic threat to food safety. DNA probes could help detect the presence of mycotoxin-producing fungi that grow under certain conditions in plant materials such as improperly dried maize and peanuts. DNA probes could also be used to learn more about the sources of fungal contamination in the environment and as a means to develop management strategies under field conditions. Given the expanding nature of these markets, a growing number of companies are developing biotechnology-based diagnostics for food and agriculture. The desire for rapid and cost effective biotechnology-based diagnostic testing processes will offer the largest challenge to the industry.

18.8.1 Biosensors

Biosensor technology combines our knowledge of biology with advances in microelectronics. The development of biosensors offers great promise for improving food processing, analysis, and safety assurance. Biosensors are detecting devices that rely on the specificity of cells and molecules to identify and measure substances at extremely low concentrations. The highly specific actions of biological molecules can be exploited for use in biosensors that can measure the concentration of specific components in complex mixtures. Biosensors may utilize either whole bacterial cells or specific molecules (e.g., enzymes, antibodies, or biomimetics) as a detection system. Combinations of biosensors in arrays can be exploited to deal with a diversity of toxicants and pollutants. These molecules and cells can be immobilized on solid surfaces, and the specific reactions they mediate can be detected by various physical and chemical means (Figure 18.18) [29]. In an electronic biosensor system, when the substance of interest interacts with the biological component, the transducer produces a digital electronic signal proportional to the concentration of the substance. Biosensors are commercially available to detect a variety of sugars, alcohols, esters, peptides, amino acids, cell types, and antibiotics. Development of tailor-made membranes capable of separating molecules based on size, electrical charge, or solubility will accelerate biosensor development as well as the exploitation of biomimetic systems.

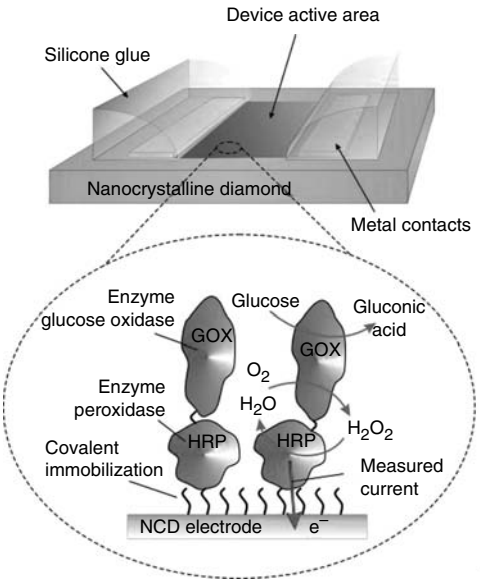


FIGURE 18.18 Schematic diagram showing the design of an enzyme-modified nanocrystalline diamond electrode. The bilayer of two enzymes, glucose oxidase and horseradish peroxidase, is covalently immobilized to the diamond surface. The cascade of electrochemical reactions initiated by the presence of glucose is converted to an electrical current measured at the diamond electrode. (*Source:* Garrido, J. A. 2006. *Biosensing with Diamond-Based Devices*. Walter Schottky Institute. http://www.wsi.tumuenchen.de/E25/research/DiamondGarrido/josegarrido/Diamond.htm)

Technologies such as microlithography, ultrathin membranes, and molecular self-assembly have the potential to facilitate the miniaturization of molecular and cellular processes for enrichment, detection, and analysis of chemical and microbiological contaminants in food. Advances in the semiconductor industry have made it possible to combine chemical and biological components and integrated circuits in miniaturized systems. Biosensors can be inserted directly into food processing streams to obtain on-line, real-time measurements of important food processing parameters. Miniature biosensors could also be incorporated into food packages to monitor temperature stress, microbial contamination, or remaining shelf life, and to provide a visual indicator to consumers of product state at the time of purchase.

Obviously, these new technologies need to be validated for application in food production. An appropriately designed long-term molecular biology study could characterize and correlate food-associated microbiological isolates in existing culture collections. Food-related isolates from these collections could be used to screen foods for microbiological contamination. Foods identified in this way then would serve as focal points for regulatory agencies and critical systems aimed at improving control over growing, shipping, processing, distribution, and other steps during which these foods may become contaminated.

18.9 SAFETY OF GM CROPS [24]

The consensus of scientific opinion and evidence is that the application of GM technology introduces no unique food/feed safety concerns and that there is no evidence of harm from those products that have been through a regulatory approval process. This conclusion has been reached by numerous national and international organizations (e.g., Food and Agriculture Organization/World Health Organization of the United Nations, Organization for Economic Cooperation and Development, EU

Commission, French Academy of Sciences, National Research Council of the National Academy of Sciences, Royal Society of London, and Society of Toxicology).

In contrast to traditionally bred crops, a rigorous safety-testing paradigm has been developed and implemented for GM crops, which utilizes a systematic, stepwise, and holistic safety assessment approach [16]. The resultant science-based process focuses on a classical evaluation of the toxic potential of the introduced novel trait and the wholesomeness of the GM crop. In addition, detailed consideration is given to the history and safe use of the parent crop as well as that of the gene donor(s). The overall safety evaluation is conducted using the process known as "substantial equivalence," a model that is entrenched in all international crop biotechnology guidelines. This provides the framework for a comparative approach to identify the similarities and differences between the GM product and an appropriate comparator that has a known history of safe use. By building a detailed profile on each step in the transformation process (from parent to new crop) and by thoroughly evaluating the significance, from a safety perspective, of any differences that may be detected between the GM crop and its comparator, a comprehensive matrix of information is constructed. This information is used to reach a conclusion about whether food or feed derived from the GM crop is as safe as food or feed derived from its traditional counterpart or the appropriate comparator. Using this approach in the evaluation of more than 50 GM crops that have been approved worldwide, the conclusion has been reached that foods and feeds derived from GM crops are as safe and nutritious as those derived from traditional crops. The lack of any proven adverse effects resulting from the production and consumption of GM crops grown on more than 235 million cumulative ha over the past 7 years supports these safety conclusions.

The U.S. National Research Council in "Genetically Modified Pest-Protected Plants: Science and Regulation" [64] determined that no difference exists between crops modified through modern molecular techniques and those modified by conventional breeding practices. The NRC emphasized that the authors were not aware of any evidence suggesting foods on the market today are unsafe to eat because of genetic modification. In fact, the scientific panel concluded that growing such crops could have environmental advantages over other crops.

In a 2003 position paper, the Society of Toxicology [82] corroborated this finding and noted that there is no reason to suppose that the process of food production through biotechnology leads to risks of a different nature than those already familiar to toxicologists or to risks generated by conventional breeding practices for plant, animal, or microbial improvement. It is therefore important to recognize that it is the food product itself, rather than the process through which it is made, that should be the focus of attention in assessing safety.

Similarly an EU Commission Report [22] that summarized biosafety research of 400 scientific teams from all parts of Europe conducted over 15 years stated that research on GM plants and derived products so far developed and marketed, following usual risk assessment procedures, has not shown any new risks to human health or the environment beyond the usual uncertainties of conventional plant breeding. Indeed, the use of more precise technology and the greater regulatory scrutiny probably make GM plants even safer than conventional plants and foods. Recent transcriptomic and metabolomic [4,11] studies in wheat and potatoes, respectively, show greater variation within and between conventionally bred cultivars and even growth locations than between GM and parental variety, except for the intended change. To reinforce this observation, differences within lines between sites were generally greater than differences between lines at the same site for both.

However, transcriptomics may be of limited value since assessing potential effects of gene expression on metabolic or phenotypic traits, since so far, understanding of biological networks is limited, prevents any valid predictions. It is better to directly measure the outcome of genotype $\times$ environment interactions instead of trying to predict potential side effects.

Metabolomics is a more valid assessment system. These applications follow a two-tiered approach: they can be used for sample discrimination and classification (e.g., for GM comparator analysis) or for biochemical and mechanistic studies. These tools can be employed to understand which parts of larger biochemical networks respond to genetic modification. Unbiased and comprehensive

assessments of potential and immediate risks should therefore focus solely on proteins and metabolite analyses, accompanied by necessary developments in validation, statistical assessments, and databases. Where compositional differences are suggested by fingerprinting studies of GM crops, the comparative assessment process, using validated and quantitative compositional analysis methods, can be guided by the fingerprinting results to reach a more informed understanding of the fingerprint results in order to determine whether additional safety assessments are needed.

The main principles of the international consensus approach are listed below [15]. They serve to illustrate the variety of principles that have been at the center of discussions and that are continuously being updated:

- *Substantial equivalence*: This is the guiding principle for safety assessment. In short, substantial equivalence involves the process of comparing the GM product to a conventional counterpart with a history of safe use. Such a comparison commonly includes agronomic performance, phenotype, expression of transgenes, and composition (macro- and micronutrients) and identifies the similarities and differences between the GM product and the conventional counterpart. Based on the differences identified, further investigations may be carried out to assess the safety of these differences. These assessments include any protein(s) which are produced from the inserted DNA.
- *Potential gene transfer*: Where there is a possibility that selective advantage may be given to an undesirable trait from a food safety perspective, this should be assessed. An example is in the highly unlikely event of a gene coding for a plant made pharmaceutical being transferred to corn. Where there is a possibility that the introduced gene(s) may be transferred to other crops, the potential environmental impact of the introduced gene and any conferred trait must be assessed.
- *Potential allergenicity*: Since most food allergens are proteins, the potential allergenicity of newly expressed proteins in food must be considered. A decision-tree approach introduced by ILSI/IFBC in 1996 has become internationally acknowledged and recently updated by Codex FAO/WHO, 2003. The starting point for this approach is the known allergenic properties of the source organism for the genes. Other recurrent items in this approach are structural similarities between the introduced protein and allergenic proteins, digestibility of the newly introduced protein(s), and eventually (if needed), sera-binding tests with either the introduced protein or the biotechnology-derived product.
- *Potential toxicity*: Some proteins are known to be toxic, such as enterotoxins from pathogenic bacteria and lectins from plants. Commonly employed tests for toxicity include bioinformatic comparisons of amino acid sequences of any newly expressed protein(s) with the amino acid sequences of known toxins with those of introduced proteins, as well as rodent toxicity tests with acute administration of the proteins. In addition to purified proteins, whole grain from GM crops has been tested in animals, commonly in subchronic (90-day) rodent studies.
- *Unintended effects*: Besides the intended effects of the modification, interactions of the inserted DNA sequence with the plant genome are possible sources of unintended effects. Another source might be the introduced trait unexpectedly altering plant metabolism. Unintended effects can be both predicted and unpredicted. For example, variations in intermediates and endpoints in metabolic pathways that are the subject of modification while undesirable are predictable, while switching on of unknown endogenous genes through random insertion in control regions is both unintended and unpredictable. The process of product development that selects a single commercial product from hundreds to thousands of initial transformation events eliminates the vast majority of situations that might have resulted in unintended changes. The selected commercial product candidate event undergoes additional detailed phenotypic, agronomic, morphological, and compositional analyses to further screen for such effects.

- *Long-term effects*: It is acknowledged that the premarket safety assessment should be rigorous to exclude potentially adverse effects of consumption of foods or feeds derived from GM crops. Nevertheless, some have insisted that such foods should also be monitored for long-term effects by postmarket surveillance. No international consensus exists as to whether such surveillance studies are technically possible without a testable hypothesis in order to provide meaningful information regarding safety, and a GM crop with a testable safety concern would most likely not pass regulatory review. The notion of using measurable biomarkers has been suggested but then these need to be determined for all foods and feeds whatever the source and balanced against reasonable economic burden.

18.9.1 Labeling

The question of whether foods derived from organisms containing rDNA should be specially labeled has received a great deal of attention. The FDA's [25] approach to the labeling of foods, including those genetically engineered or otherwise novel, is that the label must be accurate and "material." There are only two situations in which the FDA can require that a transgenic origin or ingredient be disclosed on the food label: (1) the FDA may mandate the disclosure of facts on a product label that relate to material consequences that can follow the consumption of a food (e.g., kidney beans that must be soaked and cooked before eating) and (2) the FDA can require that a label reveal facts necessary to correct or balance other representations made by the manufacturer or seller. Accordingly, labeling is required "if a food derived from a new plant variety differs from its traditional counterpart such that the common or usual name no longer applies, or if a safety or usage issue exists to which consumers must be alerted." The policy statement also emphasizes that no premarket review or approval is required unless characteristics of the biotech food explicitly raise safety issues, and that—in as much as the genetic method used in the development of a new plant variety does not meet either of the two criteria for "materiality"—the FDA cannot require the labeling to include this information. Obviously, many of the novel nutritionally enhanced foods expected on the market in the next few years will be labeled as they will differ from their traditional counterparts and in most instances, the company marketing them will want to proclaim their enhanced nutritional value.

18.9.2 Molecular Pharming

Although the use of plants to produce pharmaceuticals is termed plant-made pharmaceuticals (PMPs) or industrial products (PMIPs) and is beyond the scope of this chapter, the issue of regulatory oversight is relevant to ensuring the integrality of the food and feed supply. Candidate plants for the production of PMPs include familiar crops such as alfalfa, canola, maize, potato, rice, safflower, soybean, and tobacco. These food and nonfood crops will be treated altogether differently than the biotechnology-derived crops designated for food purposes. Issues of safety and efficacy are evaluated when considering candidacy plants. Candidate pharmaceutical producing plants have been studied with respect to pollination, genetics, seed dormancy, and weediness potential. This information can be useful for addressing several concerns, including pollen movement and subsequent gene flow between conventionally bred and biotechnology-derived crops. A long history of cultivation shows that the candidate crops are the least likely to be invasive of "natural" ecosystems. This information will be used to ensure maximal isolation of the plants from food producing crops.

The infrastructure of regulation has been in place for nearly a decade, and it continues to evolve as experience with biotechnology-derived food crops grows. Risk management must necessarily focus on providing protection for human health (worker and consumer) and ecosystems. The principle concerns are for gene flow and comingling. There are several methods to control gene flow with relative levels of effectiveness. Some strategies to reduce the risk of gene flow from transgenic crops, such as the use of male sterile plants, work well but are limited to a few species. For the many crops in which chloroplasts are strictly maternally inherited, which is to say not transmitted through

pollen, transformation of the chloroplast genome should provide an effective way to contain foreign genes. In the past few years, patents have been issued for techniques linking "suicide" genes to DNA "switches" that can be tripped inside pollen cells, impeding their development, and also issued for techniques based on genes that kill off hybrid seeds as they attempt to germinate. These Genetic Use Restriction Technologies, or GURTs (pejoratively termed "terminator technology"), of which there are several, basically render crop fertility into a trait that can be switched on or off with messengers.

Tests to date show even when cross-pollination in maize has occurred, the probability of finding one transgenic seed in thousands of seeds is low. It is reasonable to conclude that under proper confinement measures, any inadvertent exposures attributable to gene flow are very low, and thus the risk of adverse effects are correspondingly low if any one is inadvertently exposed through their food to an active pharmaceutical ingredient (API). However, in this business perception is reality and the discovery, or indeed deliberate adulteration, of the food supply despite low or no true risks could potentially scupper the whole promising industry.

18.10 CONCLUSION

The proper balance of safety testing by companies commercializing biotech products and governmental regulation is a legitimate area for further debate, as are environmental safeguards. But the purpose of such debate should be to improve biotech research and enhance risk assessment, not stop all progress. The most that can be expected of any oversight regimen is that foods developed using all methods should receive the same level of evaluation both with regard to impact on the environment and safety to the consumer. Millions of people have already eaten the products of genetic engineering and no adverse effects have been demonstrated due to the techniques per se. Both current science and long-term experience support the repeated conclusions of learned bodies that it should be the product, not the process by which it is developed, that should be evaluated for both risk and benefit. Scientists are confident that if we abandon the scientific method in judging the safety of the food supply and the impact on the environment, we will slow or destroy the advances that will reduce the use of agricultural chemicals and less safe agricultural practices in this country and we will limit the potential of improved nutrition and quality that promise to strengthen the agriculture economies in the United States and around the world.

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Part IV

Appendices

Appendix A: International System of Units (SI): The Modernized Metric System

System of unit is the form of the metric system that is preferred for all applications. All units associated with the various cgs systems (measurement systems based on centimeter, gram, and second) are to be avoided. The primary source of the following information is Reference 2, but two English translations [1,5] of the primary source and two ISO publications [3,4] were also used to prepare this appendix.

THREE CLASSES OF SI UNITS

Base Units

These well-defined units are regarded as dimensionally independent, and only one base unit is defined for each basic quantity.

	SI Base Unit	
Quantity[a]	**Name**	**Symbol**[b]
Length	Meter	m
Mass	Kilogram	kg
Time	Second	s
Electric current	Ampere	A
Thermodynamic temperature[c]	Kelvin	K
Amount of substance	Mole	mol
Luminous intensity	Candela	cd

[a] Measurable attributes of phenomena or matter.
[b] Unit symbols do not change in the plural.
[c] Use of degree Celcius (°C) is common and this is the SI unit used for the Celsius scale. $°C = T - 273.15$, where T is temperature in kelvin. The unit "Celsius" is equal to the unit "kelvin." An interval or difference in temperature can be expressed in either kelvin or degree Celsius, the former being customary in all thermodynamic expressions.

Supplementary SI Units

	SI Suppementary Unit	
Quantity	**Name**	**Symbol**
Plane angle	Radian	rad
Solid angle	Steradian	sr

Derived Units

Derived units are formed by combining base units, supplementary units, and other derived units, and are expressed algebraically in accordance with the relations existing among the corresponding quantities. The derived units are said to be coherent when they do not contain factors other than one. Because base units and coherently derived units do not always have a convenient size, subunits are sometimes constructed by using factors other than one (e.g., milligrams per cubic meter). These derived units are said to be noncoherent and the accepted prefixes and symbols are shown later in this section. Some coherently derived units along with their special names are listed next.

	SI Derived Unit			
Quantity[a]	**Name**	**Symbol**	**Expression in SI Base Units**	**Expression in Other Units**
Activity (of a radionuclide)	Becquerel	Bq	s^{-1}	
Area	Square meter	m^2		
Celsius temperature	Degree Celsius	°C	See footnote c of base unit table	
Dose (absorbed)	Gray	Gy	$m^2 \cdot s^{-2}$	J/kg
Dose rate (absorbed)	Gray per second	Gy/s	$m^2 \cdot s^{-3}$	
Electric conductance	Siemens	S	$A^2 \cdot s^3 \cdot kg^{-1} \cdot m^{-2}$	A/V
Electric field strength	Volt per meter	V/m	$m \cdot kg \cdot s^{-3} \cdot A^{-1}$	
Electric potential, potential difference, electromotive force	Volt	V	$kg \cdot m^2 \cdot s^{-3} \cdot A^{-1}$	W/A
Electric resistance	Ohm	Ω	$kg \cdot m^2 \cdot A^{-2} \cdot s^{-3}$	V/A
Electricity (quantity), electric charge	Coulomb	C	$A \cdot s$	
Energy, work, quantity of heat	Joule	J	$kg \cdot m^2 \cdot s^{-2}$	$N \cdot m$
Entropy	Joule per kelvin	J/K	$m^2 \cdot kg \cdot s^{-2} \cdot K^{-1}$	
Force	Newton	N	$kg \cdot m \cdot s^{-2}$	
Frequency (of a periodic phenomenon)	Hertz	Hz	s^{-1}	
Heat capacity	Joule per kelvin	J/K	$m^2 \cdot kg \cdot s^{-2} \cdot K^{-1}$	
Heat flux density	Watt per square meter	W/m^2	$kg \cdot s^{-3}$	
Mass density	Kilogram per cubic meter	kg/m^3		
Molar energy	Joule per mole	J/mol	$m^2 \cdot kg \cdot s^{-2} \cdot mol^{-1}$	
Molar entropy	Joule per mole kelvin	J/(mol K)	$m^2 \cdot kg \cdot s^{-2} \cdot K^{-1} \cdot mol^{-1}$	
Molar heat capacity	Joule per mole kelvin	J/(mol K)	$m^2 \cdot kg \cdot s^2 \cdot K^{-1} \cdot mol^{-1}$	
Moment of force	Newton meter	N m	$m^2 \cdot kg \cdot s^{-2}$	
Permittivity	Farad per meter	F/m	$m^{-3} \cdot kg^{-1} \cdot s^4 \cdot A^2$	

(Continued)

	SI Derived Unit			
Quantity[a]	**Name**	**Symbol**	**Expression in SI Base Units**	**Expression in Other Units**
Power, radiant flux	Watt	W	$kg \cdot m^2 \cdot s^{-3}$	J/s
Pressure, stress	Pascal	Pa	$kg \cdot m^{-1} \cdot s^{-2}$	N/m^2
Specific heat capacity	Joule per kilogram kelvin	J/(kg K)	$m^2 \cdot s^{-2} \cdot K^{-1}$	
Speed, velocity	Meter per second	m/s		
Surface tension	Newton per meter	N/m	$kg\ s^{-2}$	
Thermal conductivity	Watt per meter kelvin	W/(m K)	$m \cdot kg \cdot s^{-3} \cdot K^{-1}$	
Viscosity, dynamic	Pascal second	Pa s	$m^{-1} \cdot kg \cdot s^{-1}$	
Viscosity, kinematic	Square meter per second	m^2/s		
Volume	Cubic meter	m^3		

[a] Measurable attributes of phenomena or matter.

PREFIX NAMES AND SYMBOLS

Prefix names and symbols representing decimal multiples and submultiples of SI units, except for kilogram, are listed below (partial listing). SI prefixes should be used to indicate orders of magnitude, thus eliminating nonsignificant digits and leading zeros in decimal fractions, and providing a convenient alternative to the powers-of-ten notation often used in computation. It is preferable to choose a prefix so that the numerical value lies between 0.1 and 1000. Care should be taken to minimize the variety of prefixes used even if this violates the previous statement.

Multiplication Factor	**Prefix**	**Symbol**
10^{12}	tera	T
10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{2}	hecto[a]	h
10^{1}	deka[a]	da
10^{-1}	deci[a]	d
10^{-2}	centi[a]	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{18}	atto	a

[a] Generally to be avoided (exceptions apply to expressions of area and volume).

SOME RULES OF STYLE AND USAGE FOR SI UNIT SYMBOLS

1. Unit symbols should be printed in upright type, generally lower case, unless the unit is derived from a proper name, when the first letter of the symbol is upper case. The exception is the symbol for liter, where either "l" or "L" is acceptable.

2. Unit symbols remain unaltered in the plural.
3. Unit symbols are not followed by a period except when used at the end of a sentence.
4. When a quantity is expressed as a numerical value and a unit symbol, a space should be left between them, for example, 35 mm, not 35mm. *Exceptions*: no space is left between the numerical value and symbols for degree, minute, and second of plane angle.
5. When a quantity is expressed as a number and a unit and is used in an adjectival manner, it is preferable to use a hyphen instead of a space between the number and the symbol, for example, a 35-mm film. *Exception*: a 60° angle.
6. Units formed by multiplication or division. For division, oblique strokes (/) or negative exponents can be used, but if oblique strokes are used, these strokes must not be repeated in the same group unless ambiquity is avoided by a parentheses (e.g., m/s/s is forbidden); m/s, $\frac{\mathrm{m}}{\mathrm{s}}$, $\mathrm{m} \cdot \mathrm{s}^{-1}$ are equally acceptable. For multiplication, two or more units may be expressed in either of the following ways: $\mathrm{Pa} \cdot \mathrm{s}$ or Pa s.
7. When names of units are used in quotients, use the word 'per' and not an oblique stroke, for example, meter per second, not meter/second.
8. To avoid ambiguity in complicated expressions, symbols are preferred over words.
9. When writing numerals less than 1, a zero should appear before the decimal marker.
10. For American usage, the dot is used for the decimal marker instead of the comma, and the spellings "meter," "liter," and "deka" are used instead of "metre," "litre," and "deca."

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Appendix B: Conversion Factors (Non-SI Units to SI Units)

To Convert from	To (SI units)	Multiply by[a]
Area		
ft^2	square meter (m^2)	9.290304*E–02
ha	square meter (m^2)	1.000000*E+04
$in.^2$	square meter (m^2)	6.451600*E–04
yd^2	square meter (m^2)	8.361274 E–01
Energy		
British thermal unit (mean)	joule (J)	1.05587 E+03
Calorie (kilogram, International Table)	joule (J)	4.186800*E+03
Calorie (kilogram, thermochemical)	joule (J)	4.184000*E+03
Electron volt	joule (J)	1.60219 E–19
Erg	joule (J)	1.000000*E–07
kW · h	joule (J)	3.600000*E+06
Force		
Dyne	newton (N)	1.000000*E–05
Kilogram-force	newton (N)	9.806650*E+00
Poundal	newton (N)	1.382550 E–01
Length		
Angstrom	meter (m)	1.000000*E–10
Foot (U.S. survey)	meter (m)	3.048006 E–01
in.	meter (m)	2.540000*E–02
Mil	meter (m)	2.540000*E–05
Mass		
Carat (metric)	kilogram (kg)	2.000000*E–04
Grain	kilogram (kg)	6.479891*E–05
Gram	kilogram (kg)	1.000000*E–03
Ounce (avoirdupois)	kilogram (kg)	2.834952 E–02
Ounce (troy or apothecary)	kilogram (kg)	3.110348 E–02
Pound (lb avoirdupois)	kilogram (kg)	4.535924 E–01
Pound (troy or apothecary)	kilogram (kg)	3.732417 E–01
Pressure or stress (force per unit area)		
Atmosphere (standard)	pascal (Pa)	1.013250*E+05
Bar	pascal (Pa)	1.000000*E+05
$Dyne/cm^2$	pascal (Pa)	1.000000*E–01
Inch of mercury (32°F)	pascal (Pa)	3.38638 E+03

(Continued)

(Continued)

To Convert from	To (SI units)	Multiply by[a]
Inch of mercury (60°F)	pascal (Pa)	3.37685 E+03
Inch of water (39.2°F)	pascal (Pa)	2.49082 E+02
Inch of water (60°F)	pascal (Pa)	2.4884 E+02
Psi	pascal (Pa)	6.894757 E+03
Torr (mmHg, 0°C)	pascal (Pa)	1.33322 E+02
Radiation units		
Curie	becquerel (Bq)	3.700000*E+10
Rad	gray (Gy)	1.000000*E–02
Rem	sievert (Sv)	1.000000*E–02
Roentgen	coulomb per kilogram (C/kg)	2.580000*E–04
Temperature		
Degree Celsius	kelvin (K)	$T_K = t_C + 273.15$
Degree Fahrenheit	degree Celsius (°C)	$t_C = (t_F - 32)/1.8$
Degree Fahrenheit	kelvin (K)	$T_K = (t_F + 459.67)/1.8$
Degree Rankine	kelvin (K)	$T_K = T_R/1.8$
Kelvin	degree Celsius (°C)	$t_C = T_K - 273.15$
Viscosity		
Centipoise (dynamic viscosity)	pascal second (Pa s)	1.000000*E–03
Centistokes (kinematic viscosity)	square meter per second (m^2/s)	1.000000*E–06
Poise	pascal second (Pa s)	1.000000*E–01
Stokes	square meter per second (m^2/s)	1.000000*E–04
Volume (includes capacity)		
ft^3	cubic meter (m^3)	2.831685 E–02
Gallon (Canadian liquid)	cubic meter (m^3)	4.546090 E–03
Gallon (U.K. liquid)	cubic meter (m^3)	4.546092 E–03
Gallon (U.S. liquid)	cubic meter (m^3)	3.785412 E–03
$in.^3$	cubic meter (m^3)	1.638706 E–05
Liter	cubic meter (m^3)	1.000000*E–03
Ounce (U.K. fluid)	cubic meter (m^3)	2.841306 E–05
Ounce (U.S. fluid)	cubic meter (m^3)	2.957353 E–05
Pint (U.S. liquid)	cubic meter (m^3)	4.731765 E–04
Quart (U.S. liquid)	cubic meter (m^3)	9.463529 E–04
yd^3	cubic meter (m^3)	7.645549 E–01

[a] Factors with an asterisk are exact.

Source: American Society for Testing and Materials (1991), *Standard Practice for Use of the International System of Units (the Modernized Metric System)*, E380-91a, ASTM, Philadelphia, PA.

Appendix C: Greek Alphabet

Greek Form			
Upper Case	**Lower Case**	**Name**	**Roman or English Equivalent**
Α	α	alpha	a
Β	β	beta	b
Γ	γ	gamma	g
Δ	δ	delta	d
Ε	ε	epsilon	e (short)
Ζ	ζ	zeta	z
Η	η	eta	e (long)
Θ	θ	theta	th
Ι	ι	iota	i
Κ	κ	kappa	k, c
Λ	λ	lambda	l
Μ	μ	mu	m
Ν	ν	nu	n
Ξ	ξ	xi	x
Ο	ο	omicron	o (short)
Π	π	pi	p
Ρ	ρ	rho	r
Σ	σ, ς	sigma	s
Τ	τ	tau	t
Υ	ϕ	upsilon	u, y
Φ	ϕ	phi	ph
Χ	χ	chi	kh, ch
Ψ	ψ	psi	ps
Ω	ω	omega	o (long)

Appendix D: Calculating Relative Polarities of Compounds Using the Fragmental Constant Approach to Predict log P Values

Relative lipophilicity evaluations are important to the field of study known as quantitative structure–activity relationships (QSAR), and help explain or account for various chemical and biological properties of chemical compounds. Such an approach is also relevant to explaining behavior of food components, which may be dependent on how they are distributed or partitioned in multiphasic, food matrices. The partition coefficient (P) is defined as "the concentration ratio of (the) same molecular species in a solvent pair" [1], typically between aqueous and organic phases. The partition coefficient can be expressed as

$$P = c_o/c_w \quad \text{or} \quad \log P = \log c_o - \log c_w$$

where c_o and c_w represent the molar concentrations of the compounds partitioned into the respective organic and aqueous phases. The relevant solvent pair in food and biological systems are buffered aqueous:biological membrane, or buffered aqueous:bulk lipid phases. Virtually any organic solvent can be used in typical QSAR studies, ranging from the more apolar ones such as cyclohexane and heptane, to the more polar solvents such as n-butanol. There is widespread acceptance of the n-octanol:water pair for QSAR studies, and this system is also relevant to component partitioning into red blood cells. Other benefits from employing octanol are low toxicity, low ultraviolet (UV) absorptivity (for analytical reasons), low volatility, and good solvent properties (including a saturation solubility of water of 2.3 M).

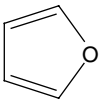
The fragmental constant approach to calculating or predicting log P values for a given component is based on the sum of the contributions of each functional group to the log P value of the chemical compound. A subset of log P values for various fragments, depending on whether they are constitutive groups of aliphatic or aromatic compounds, is provided.

Aliphatic Fragment	Log *P*	Aromatic Fragment	Log *P*	Heterocyclic Groups and Solvents	Log *P*
CH_3	0.724	C_6H_5	1.902	Furyl	1.086
CH_2	0.519	C_6H_4	1.697	Benzofuryl	2.374
CH	0.315	C_6H_3	1.493	Pyrrolyl	0.615
$CH_2{=}CH$	0.834	C_6H_2	1.288	Indolyl	1.902
$CH{\equiv}C$	0.425	C_6H	1.084	Imidazolyl	−0.046
C	0.110	C	0.110		
				Water	−1.38
OCH_3	−0.821	OCH_3	0.274	Dimethylsulfoxide	−1.35
COOH	−0.942	COOH	−0.066	Dimethylformamide	−1.01
COH	−0.990	COH	−0.333	Methanol	−0.77
$CONH_2$	−2.011	$CONH_2$	−1.135	Acetonitrile	−0.34
CONH	−2.435	CONH	−1.559	Ethanol	−0.31
COO	–1.200	COO	−0.543	Acetone	−0.24
COO^-	−4.967	COO^-	−4.091	Ethyl acetate	0.73
CO	−1.633	CO	−0.976	*n*-Butanol	0.88
OH	−1.448	OH	−0.353	Diethyl ether	0.89
NH_2	−1.340	NH_2	−0.902	Chloroform	1.97
NH	−1.814	NH	−0.938	Methylene chloride	1.25
N	−2.074	N	−0.979	Toluene	2.73
SH	−0.046	SH	0.611	*n*-Octanol	3.00
S	−0.558	S	0.099	Cyclohexane	3.44
SS	0.322	SS	NA	Hexane	3.90
O	−1.595	O	−0.450	Heptane	4.66
NO_2	−0.915	NO_2	−0.039	Dodecanol	5.13
H	0.204	H	0.204	Hexadecane	8.25

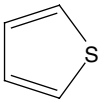
Log *P* values for fragments were obtained from Reference 1. Log *P* values for solvents were obtained from Reference 2.

EXAMPLES OF LOG *P* VALUES OF SELECTED FOOD CHEMICAL COMPONENTS AND BASE STRUCTURES

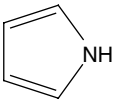
1. Furan
CAS 110-00-9
Exp log *P* 1.34
Calc log *P* 1.248–1.290



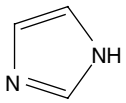
2. Thiophene
CAS 110-02-1
Exp log *P* 1.61
Calc log *P* 1.707–1.817



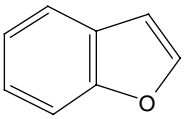
3. Pyrrole
CAS 109-97-7
Exp log *P* 0.75
Calc log *P* 0.760–0.819



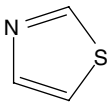
4. Imidazole
CAS 288-32-4
Exp log *P*–0.08
Calc log *P* 0.083–0.158



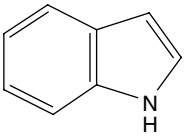
5. Benzofuran
CAS 271-89-6
Exp log *P* 2.67
Calc log *P* 2.536–2.578



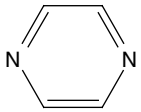
6. Thiazole
CAS 288-47-1
Exp log *P* 0.44
Calc log *P* 0.504



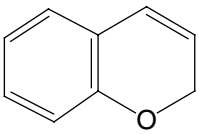
7. Indole
CAS 120-72-9
Exp log *P* 2.14
Calc log *P* 2.048–2.106



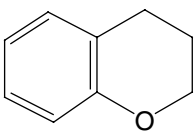
8. Pyrazine
CAS 290-37-9
Exp log *P*–0.26
Calc log *P*–0.260



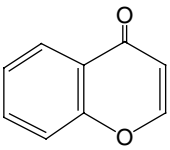
9. Benzopyran
CAS 254-04-6
Est log *P* 2.78



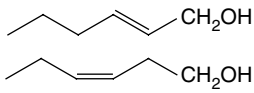
10. Chroman
CAS 493-08-3
Est log *P* 3.00



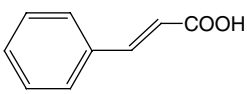
11. Benzopyrone
CAS 491-38-3
Exp log *P* 1.33



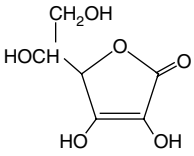
12. Hexenols (2*t* or 3*c*)
CAS 928-(95-0)(96-1)
Est log *P* 1.61 (both)



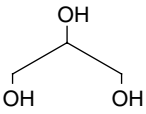
13. Cinnamic Acid
CAS 140-10-3
Exp log *P* 2.13
Calc log *P* 2.08



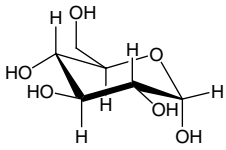
14. Ascorbic Acid
CAS 50-817
Exp log *P*–1.85



15. Glycerol
CAS 56-81-5
Exp log *P*–1.76



16. Glucose
CAS 50-99-7
Exp log *P*–3.24
Calc log *P*–2.98

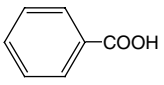


Exp log *P* and Est log *P* values represent experimentally determined and estimated values from experimental data, respectively [2]. Calc log *P* values represent those determined from the fragmental constant approach [1]. CAS numbers refer to the Chemical Abstract Service (CAS) Registry Number, which may be found in the Merck Index and many chemical catalogs or at http://www.syrres.com/esc/datalog.htm.

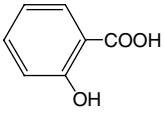
EXAMPLES OF CALCULATED LOG *P* VALUES BASED ON FRAGMENTAL CONSTANT APPROACH

The following compounds, representative of various types of antioxidant components, are selected to contrast estimated or experimentally determined log *P* values [2] with calculated log *P* values based on fragmental constant approach.

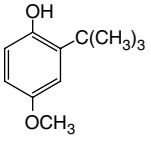
Benzoic Acid
CAS 65-85-0
Exp log *P* 1.87
Calc log *P* (below)
$1 \times C_6H_5[1.902]+$
$1 \times COOH(Ar)$
$[-0.066] = 1.836$



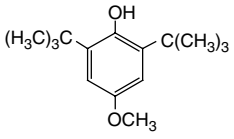
Salicylic Acid
CAS 69-72-7
Exp log *P* 2.26
Calc log *P* (below)
$C_6H_4[1.697] + COOH(Ar)$
$[-0.066] + OH(Ar)$
$[-0.353] = 1.278$



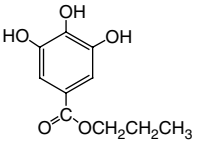
BHA
CAS 25013-16-5
Exp log *P* 3.50
Calc log *P* (below)
$C_6H_3[1.493] + C[0.110]+$
$OCH_3(Ar)[0.274]+$
$CH_3[3 \times 0.724] = 4.05$



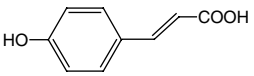
BHT
CAS 128-37-0
Exp log *P* 5.10
Calc log *P* (below)
$C_6H_2[1.288]+$
$C[2 \times 0.110]+$
$OCH_3(Ar)[0.274]+$
$CH_3[6 \times 0.724] = 6.13$



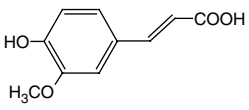
n-Propyl Gallate
CAS 121-79-9
Exp log *P* 1.80
Calc log *P* (below)
$C_6H_2[1.288] + OH(Ar)$
$[3 \times -0.353] + COO(Ar)$
$[-0.543] + CH_3[0.724]+$
$CH_2[2 \times 0.519] = 1.45$



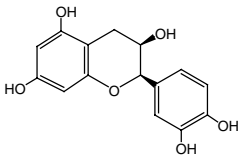
p-Coumaric Acid
CAS 501-98-4
Exp log *P* 1.46
Calc log *P* (below)
$C_6H_4[1.697] + COOH(Al)$
$[-0.942] + OH(Ar)[-0.353]+$
$CH[2 \times 0.315] =$
1.03



Ferulic Acid
CAS 537-98-4
Exp log *P* 1.42
Calc log *P* (below)
$C_6H_3[1.493] + COOH(Al)$
$[-0.942] + OH(Ar)[-0.353]+$
$CH[2 \times 0.315]+$
$OCH_3[0.274] = 1.10$



(−)Epicatechin
CAS 490-46-0
Est log *P* 1.18
Calc log *P* (below)
Chroman [3.00]−
$H[4 \times 0.204]+$
$C_6H_3[1.493]+$
$OH(Ar)[4 \times -0.353]+$
$OH(Al)[-1.448] = 0.82$



Exp log *P* and Est log *P* values represent experimentally determined and estimated values from experimental data, respectively [2]. Calc log *P* values represent those determined from the fragmental constant approach based on the first table appearing in this appendix [1].

OTHER EXAMPLES OF CALCULATION OF LOG *P* VALUES

The following compounds are selected to contrast estimated or experimentally determined log *P* values [2] with calculated log *P* values based on fragmental constant approach.

Leucine (CAS 61-90-5; Exp log *P* − 1.70)
Calc log *P*: COOH(Al) [−1.200]+NH_2[−1.340]+CH_3[2×0.724]+CH_2[0.519]+CH[2×0.315] = 0.057

Isoleucine (CAS 73-32-5: Exp log *P* –1.52)
Calc log *P*: COOH(Al) [−1.200]+NH_2[−1.340]+CH_3[2×0.724]+CH_2[0.519]+CH[2×0.315] = 0.057

Palmitic acid (CAS 57-10-3: Exp log *P* 7.17)
Calc log *P*: COOH(Al) [−0.942] + CH_3[0.724] + CH_2[14 × 0.519] = 7.05

Tripalmitin (CAS 555-44-2: Est log *P* 20.99)
Calc log *P*: gly-CH_2[2 × 0.519] + gly-CH[0.315] + COO(Al)[3 × −1.200] + CH_3[3 × 0.724] + CH_2[42 × 0.519] = 21.72

Ascorbyl 6-*O*-palmitate (CAS 137-66-6: Est log *P* 6.00)
Calc log *P*: ascorbic acid [−1.85] − H[0.204] + COO(Al)[−1.200] + CH_3[0.724] + CH_2[14 × 0.519] = 4.74

COMMENTARY

The above examples are intended to illustrate that relative polarity of various food chemical components can be easily estimated using the fragmental constant approach to lipophilicity of constitutive functional groups. It can be noted that the actual or experimentally determined log *P* values are often slightly different from the calculated values. These differences are attributable to anomalies that exist because of the extension of conjugated systems, steric factors, proximity of electronegative groups, hydrogen-bonding, and H atoms attached to electronegative groups. The magnitude of the discrepancy of log *P* values for leucine and isoleucine are likely caused by the ionic nature of the amine and carboxylic acid group at physiological pH as well as the interaction effects of proximal electronegative groups. Corrections can be applied to such factors (as multiples of a C_M value or "magic constant," based on each factor), but the nature of making such corrections are beyond the scope of this appendix, although they are used for the calculation of log *P* values [1] for compounds 1–6, 13 and 16 in the preceding tables. Despite the slight differences in calculated and experimental log *P* values, the fragmental constant approach to calculating log *P* values is useful for estimated relative differences in polarity among groups of structurally related compounds.

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