Chapter 2

Fruits and Vegetables

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Chapter Outline

I. INTRODUCTION

Characteristics of fruits and vegetables such as flavor, color, size, shape, and absence of external defects ultimately determine their acceptance by consumers. The development of these characteristics is the result of many chemical and biochemical changes that occur following harvesting and storage. Since harvesting fruits and vegetables at their correct stage of maturity is critical for the development of a highly acceptable product for the fresh market, or for processing, it is important to understand more fully what changes are taking place. This chapter will highlight those changes occurring within fruits and vegetables during the postharvest period. It is during this period that fruits and

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vegetables show a gradual reduction in quality concurrent with transpiration and respiration, as well as with other biochemical and physiological changes. Ultimately the plant material deteriorates because of the undesirable enzyme activity and spoilage microorganisms.

The growth and maturation of fruits and vegetables are dependent on photosynthesis and absorption of water and minerals by the parent plant. Once detached, however, they are independent units in which respiratory processes play a major role. This chapter will focus on those changes in postharvest fruits and vegetables that affect quality.

II. RESPIRATION

Respiration is the fundamental process whereby living organisms carry out the exothermic conversion of potential energy into kinetic energy. In higher plants the major storage products are sucrose and starch. These are completely oxidized in the presence of oxygen to carbon dioxide and water, with the production of adenosine triphosphate (ATP):

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{energy (heat and ATP)}$$

The latter is the form in which energy is stored within the cell. The contribution of proteins and lipids to plant respiration is difficult to assess but can occur via the formation of acetyl-coenzyme A (CoA). In the absence of oxygen, anaerobic respiration occurs, resulting in only a partial degradation of carbohydrates and a lower ATP production.

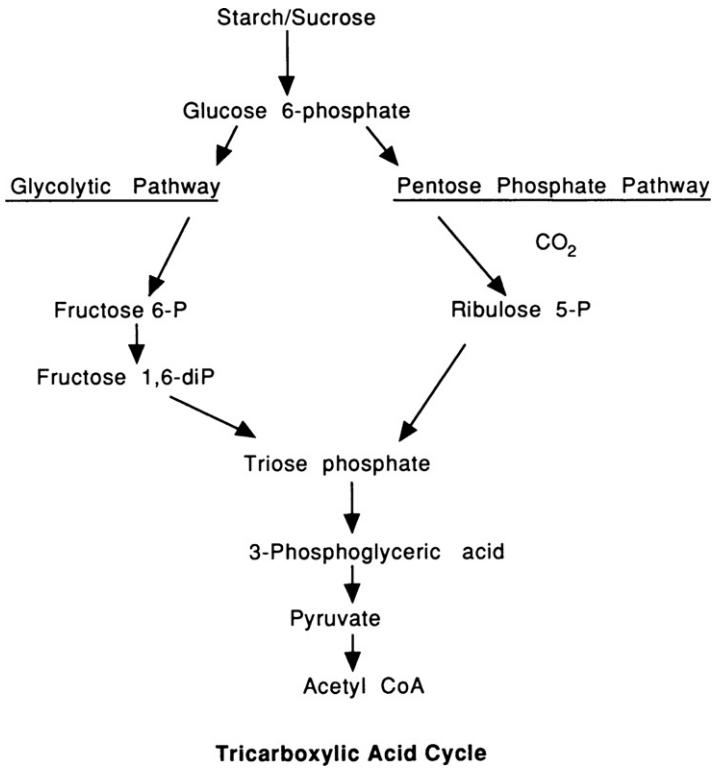
The metabolic pathways involved in the respiration of plant tissue result in the conversion of starch or sucrose to glucose-6-P. The latter is then oxidized by glycolysis (Embden−Meyerhoff pathway) or the pentose phosphate pathway to triose phosphate, which enters the tricarboxylic acid cycle by way of pyruvate (Scheme 2.1) (ap Rees, 1977). Finally, in a third stage, oxidative phosphorylation converts NADH and $FADH_2$ into chemical energy in the form of ATP (Browse *et al.*, 2006).

The contribution of these two major pathways of carbohydrate oxidation to plant respiration remains unresolved. Difficulties were encountered with the experimental techniques used in assessing the relative roles of these pathways based on the production of $^{14}CO_2$ or labeled intermediates from labeled hexoses (ap Rees, 1980). Evidence shows that both pathways exist in plant tissues (ap Rees, 1974) and that they change considerably during plant development (ap Rees, 1977). Current evidence supports the glycolytic pathway as the predominant one operating, while the maximum contribution of the pentose phosphate pathway may not exceed 30% of the total (ap Rees, 1980). The relative importance of these pathways probably depends on the particular plant, the organ, and the state of maturity.

Respiration rates of fruits and vegetables are affected by many environmental factors. In cases where this leads to negative effects on plant tissue it is defined as stress. During the storage of fruits and vegetables (Section VIII) effects of low temperatures, reduction in oxygen (O_2) concentration and increase in carbon dioxide (CO_2) concentration in the storage atmosphere are utilized to extend the storage life of produce. However, maintaining an adequate energy status is required to prevent browning or senescence of harvested fruits and vegetables (Saquet *et al.*, 2000, 2003a; Xuan *et al.*, 2005; Song *et al.*, 2006; Jiang *et al.*, 2007). It is well established that lowering of O_2 concentrations during controlled atmosphere storage reduces respiration rates and energy supply and that severe limitations of O_2 induce fermentative (anaerobic) respiration and metabolism in stored produce (Scheme 2.1). The net yield of ATP during anaerobic respiration is only 2 moles of ATP of hexose sugar compared with 36 moles of ATP per mole of hexose in aerobic respiration. Hence, energy status may be insufficient and provoke storage disorders (Jiang *et al.*, 2007).

A. Fruits

A large number of fruits exhibit a sudden sharp rise in respiratory activity following harvesting, referred to as the climacteric rise in respiration. This phenomenon was first noted by Kidd and West (1922, 1930a) as an upsurge in carbon dioxide gas at the end of the maturation phase of apples. Since then there have been numerous reports on this phenomenon in a wide range of fruits. The appropriateness of the term climacteric was questioned by Rhodes (1970), who suggested that it should be all inclusive and describe the 'whole of the control phase in the life of fruit triggered by ethylene and the concomitant changes occurring'. McGlasson *et al.* (1978), however, suggested that respiratory climacteric was the more appropriate term to describe this gaseous phenomenon. Biale and Young (1981) nevertheless still preferred the more inclusive description in which climacteric defined those physical, chemical, physiological, and metabolic changes associated with the increased rate of respiration covering the transition phase from growth and maturation to the final stages of senescence. Essentially, climacteric defines the last stages of the fruit at the cellular level, which determine the quality of the fruit that is shipped to the consumer.



SCHEME 2.1 Glycolytic and pentose phosphate pathways.

Biale (1960a, b) tentatively classified fruits as either climacteric or non-climacteric according to their respiratory rates. A later review by Biale and Young (1981), however, suggested a more extensive list of fruits from both groups as shown in Table 2.1. Eventually fruits such as cantaloupe, honeydew melon, and figs were included, all of which are considered climacteric (Lyons *et al.*, 1962; Pratt and Groeschel, 1968; Marei and Crane, 1971). A few rare fruits were also added, namely, breadfruit (Biale and Barcus, 1970), guavas, and mammee apples (Akamine and Goo, 1978, 1979a, b; Saltveit, 2004).

TABLE 2.1 Respiratory Activity of Selected Fruit

Climacteric	Non-climacteric
Apple	Blueberry
Apricot	Grape
Avocado	Grapefruit
Banana	Java plum
Breadfruit	Lemon
Fig	Olive
Guava	Orange
Mammee apple	Pineapple
Muskmelon cantaloupe	Strawberry
	Honeydew melon

Adapted from Biale and Young (1981).

The period immediately prior to the climacteric rise, when the respiratory level is at a minimum, is known as the preclimacteric. Following the completion of the climacteric rise is the postclimacteric phase, in which a decline

in the respiratory rate occurs. Unlike the sudden rise in respiratory activity which characterizes climacteric fruits, non-climacteric fruits exhibit a steady fall in respiratory activity. This downward trend in the respiratory activity was originally observed for lemons stored at 15°C by Biale and Young (1947) and later in oranges (Biale, 1960a, b). Figure 2.1 illustrates the difference in respiratory activity between climacteric and non-climacteric fruits, for example, avocados and lemons.

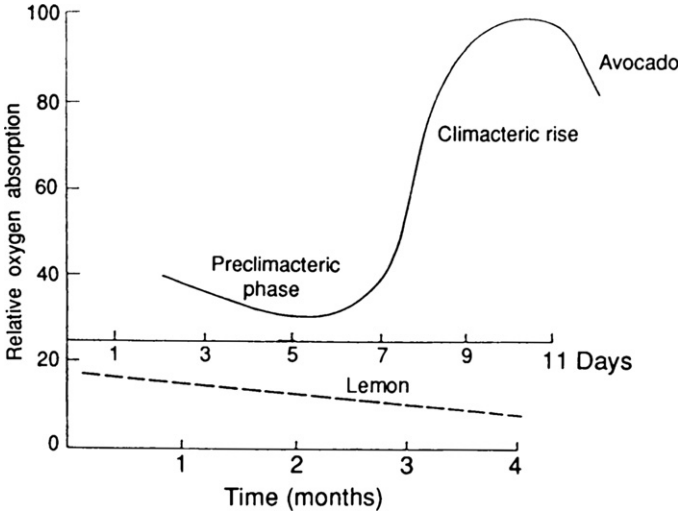


FIGURE 2.1 Respiratory trends in climacteric fruit, exemplified by the avocado, compared with non-climacteric fruit, depicted by the lemon (Biale *et al.*, 1954). *[Reprinted with permission of the copyright owner, American Society of Plant Physiology (ASPP).]*

In the original classification by Biale (1960a, b), oranges were classified as non-climacteric fruits since they had a low rate of respiration. It was soon evident that some members of the citrus fruit family had respiratory activities similar to those of climacteric fruits. In spite of the higher respiratory rates, a downward trend was observed for both 'Valencia' and 'Washington Navel' cultivars. This decline in respiration was observed by Bain (1958) in 'Valencia' oranges from fruit set to maturity. Nevertheless, Trout *et al.* (1960) reported a typical respiratory rise in oranges stored at 4.3−10°C. Their results were attributed by Biale and Young (1981) to possible chilling injury at the lower storage temperatures. Only half of those oranges kept at 10°C exhibited a rise in respiration following harvesting, which could be due to the immaturity of some of these fruits. Aharoni (1968) also used the term climacteric to describe the increase in respiratory rate in postharvest, young, unripe 'Washington Navel', 'Shamouti', and 'Valencia' oranges, as well as in Marsh's seedless grapefruit stored at 16°C and 20°C. In contrast, the full-sized and mature fruit did not exhibit any rise in respiratory activity. Eaks (1970) examined the respiratory patterns of several species of citrus fruit throughout ontogeny. The small and immature oranges and grapefruit exhibited a rise in respiration and ethylene activity when stored at 20°C for several days after harvest. As the weight of the fruit increased, characteristic of maturation, the level of carbon dioxide and ethylene production decreased until full maturity was approached or attained, at which point no change in respiration was noted. Based on this study it was evident that citrus fruits were correctly classified as non-climacteric. Rhodes (1970) noted that had the climacteric been defined as the period of enhanced metabolic activity during the transition from the growth phase to senescence in fruits, the confusion with citrus fruits could have been avoided. A similar situation was observed in grapes, in which a respiratory rise was reported by Peynaud and Riberau-Gayon (1971) to accompany rapid growth, which was referred to as 'rudimentary climacteric'. However, this was resolved when the postmaturation change in respiratory activity showed a typical non-climacteric pattern consistent with earlier work by Geisler and Radler (1963). A similar controversy arose with respect to the pineapple and while Dull *et al.* (1967) found a slight upward respiratory trend it was not typical of climacteric fruit. The identification of several tomato mutants by Herner and Sink (1973) without any climacteric pattern was later reviewed by Tigchelaar *et al.* (1978a, b). These mutants were unable to produce ethylene and had low levels of carotenoids. A particular feature was the extremely low levels of polygalacturonase, which accounted for their prolonged firmness.

B. Vegetables

Once the vegetable becomes detached from the parent plant, metabolism continues to take place, although it is the catabolic reactions that soon become dominant. The climacteric rise in respiration characteristic of certain fruits such

as apples and avocados is not apparent in vegetables, where there is no clear-cut division between maturation and breakdown.

The intensity and rate of respiration vary with the particular plant, the degree of maturity, and whether the vegetable is actively growing at the time of harvest or functioning as a storage organ. For example, McKenzie (1932) reported higher respiration intensity in freshly harvested immature lettuce (*Lactuca sativa*) during the first 12 hours, which then fell to the same level as that of mature lettuce. The deterioration of several vegetables was examined by Platenius (1942), who found that the initial respiratory rate for asparagus (*Asparagus officinalis*) at 24°C was almost 50 times greater than for potatoes. The respiration rate declined for all vegetables during 60 days' storage irrespective of the temperature. The initial rates of respiration appeared to be a useful indicator of the potential storage life of the crop during precooling and early storage. A high respiratory rate, however, was indicative of a short storage life, while the reverse was true for crops with a low respiratory rate. This is illustrated for a number of vegetables in Figure 2.2.

Vegetables and fruits can be classified as extremely high, very high, high, moderate, low, or very low according to the respiration rates (Saltveit, 2004). For example, young tissues, such as growing parts of asparagus or developing seeds of green peas, have high respiration rates, while low rates are evident in storage organs such as stems (potatoes) and bulbs (onions). Leafy vegetables appear to be moderate, while some vegetables such as cabbage can be stored at a low temperature for considerable periods. Other vegetables, including cucumbers (*Cucumis sativa*), are particularly susceptible to chilling injury if stored over a temperature range of 0−10°C (Eaks and Morris, 1956). Many other commodities, in particular those originating in the tropics or subtropics, are prone to chilling injuries when their temperature falls below 10−12°C (Wang, 1989; Saltveit, 2004). Chilling injury is diagnosed by an increase in respiration in which a plateau is reached corresponding to chilling, after which there is a decline in respiration.

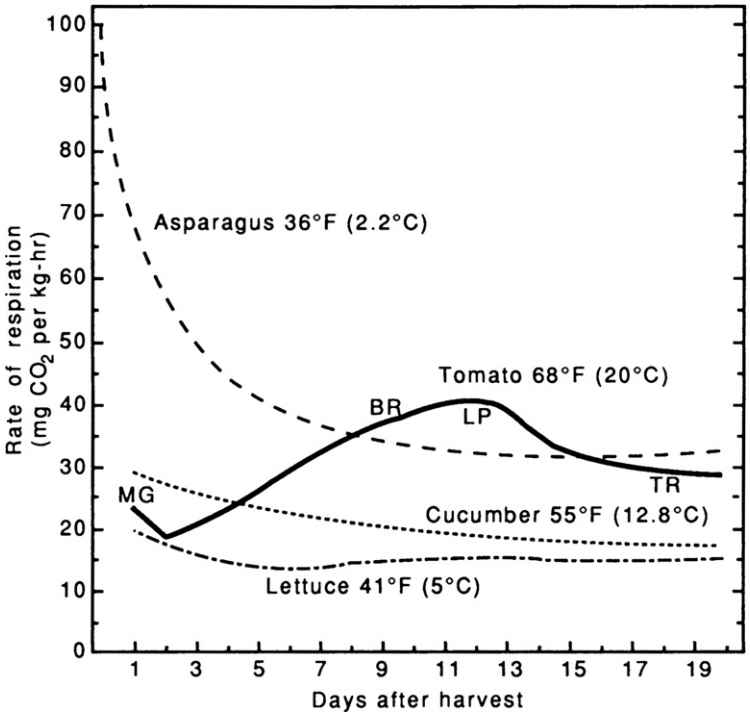


FIGURE 2.2 Rate of respiration of a shoot (asparagus), a leafy vegetable (head lettuce), a non-ripening fruit (cucumber), and a ripening fruit (tomato) at temperatures commonly encountered during their marketing (for tomato: MG = mature green; BR = breaker; LP = light pink; TR = table red) (data taken from Lipton, 1977; Pratt *et al.*, 1954; Workman *et al.*, 1957) (Ryall and Lipton, 1979).

1. Control of the Climacteric Rise

The dramatic upsurge in respiratory activity associated with the climacteric has been attributed to a number of different factors. One theory proposed was the breakdown in 'cell membrane permeability or organization resistance' (Solomos and Laties, 1973). While such changes are evident during ripening, the question remains as to whether they are the cause or the consequence of the ripening process (Theologis and Laties, 1978). The second theory focused on increased protein synthesis as a necessary prerequisite for the ripening process (Brady *et al.*, 1976; Richmond and Biale, 1966) or enhanced ATP turnover with respiratory stimulation (Biale, 1960b). Subsequent research, however, showed there was no difference in the respiratory capacity of mitochondria obtained from preclimacteric and

climacteric avocado tissue (Biale, 1969). In addition, the respiratory rate of uncoupled preclimacteric avocado slices was quite sufficient to facilitate the climacteric rise (Millerd *et al.*, 1953). This was confirmed in studies using isolated mitochondria from avocado (Biale *et al.*, 1957). Further research by Lance *et al.* (1965) and Hobson *et al.* (1966), using improved techniques, isolated mitochondria from avocado throughout all stages of the climacteric and found that the oxidative and phosphorylating activities remained unchanged provided all cofactors were present. The stimulation of glycolysis in avocado fruit under anaerobic conditions also demonstrated considerable latent glycolytic capacity (Solomos and Laties, 1974). These studies indicated the adequacy of the enzymes in preclimacteric fruits to sustain the respiratory climacteric. This was consistent with studies by Frenkel *et al.* (1968) and McGlasson *et al.* (1971), who found that while inhibiting protein synthesis prevented the ripening of intact peas and banana slices, it did not decrease the upsurge in respiration.

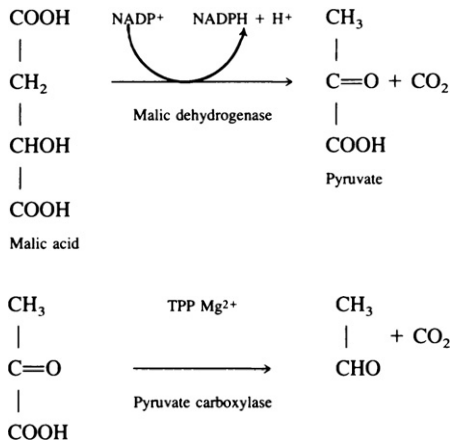
The dramatic burst in respiratory activity that accompanies the ripening of climacteric-type fruits appears to be due to some change in the mitochondrial respiratory function *in vivo* (Biale, 1960a, b). A possible explanation for this was attributed to an increase in cyanide-resistant respiration (Solomos and Laties, 1976; Solomos, 1977). Solomos and Laties (1974, 1976) observed that cyanide initiated identical physiological and biochemical changes in avocado and potato tubers. Cyanide is a known inhibitor of cytochrome oxidase, the terminal oxidase in the electron transport system. Thus, there is present a cyanide-insensitive pathway which permits the aerobic oxidation of respiratory substrates in the presence of cyanide (Bendall and Bonner, 1971). This cyanide-resistant pathway or alternative pathway was reported to be present in ethylene-responsive fruits (Solomon and Laties, 1974, 1976). Further research by Theologis and Laties (1978) led to an examination of this pathway in the respiration of ripening avocados and bananas. These researchers found that the surge in respiration during the climacteric in the intact fruit was cytochrome mediated. The preclimacteric fruit had the capacity to sustain electron transport through the cytochrome pathway, although it remained unexpressed. During the climacteric rise, the alternative pathway appears to remain at a low level of activity and may be involved in the generation of peroxide (Rich *et al.*, 1976). The enzyme responsible for the cyanide-resistant respiration is called the alternative oxidase (Vanlerbergh and McIntosh, 1997). The function of the alternative pathway is not completely resolved, but it may diminish detrimental effects of stress on respiration (Tucker, 1993; Wagner and Krab, 1995; Møller, 2001). A discussion of the possible regulatory role of cyanide in ethylene biosynthesis can be found in Section IV.

2. Enzymatic Control

The possibility of enzymatic activity being the controlling factor in the climacteric has been suggested. Tager and Biale (1957) noted a rise in carboxylase and aldolase activity during the ripening of bananas accompanied by a shift from the pentose phosphate to the glycolytic pathway. This may occur during the transition period from the preclimacteric to the postclimacteric phase in fruit ripening.

a. Malic Enzyme

Hulme *et al.* (1963) reported a sharp increase in the activities of malic enzyme and pyruvate carboxylase during the ripening of apples. This explained the slight uptake of oxygen during ripening of apples compared to the marked increase in carbon dioxide evolution.



Several studies showed that malic enzyme exhibited varying degrees of cyanide insensitivity depending on the activity of the enzyme (Lance *et al.*, 1967; Macrae, 1971; Coleman and Palmer, 1972; Neuburger and Douce, 1980). Moreau and Romani (1982) examined the oxidation of malate during the climacteric rise in avocado mitochondria with particular focus on the cyanide-insensitive alternative pathway. The increase in malic enzyme activity paralleled the increase in malate oxidation as ripening advanced through the climacteric. Malate is oxidized by malic dehydrogenase through the cytochrome pathway. It can also be oxidized by the malic enzyme, which involves the alternative pathway via a rotenone-insensitive NADH dehydrogenase located in the inner layer of the mitochondrial membrane (Palmer, 1976; Marx and Brinkmann, 1978; Rustin *et al.*, 1980). These researchers concluded that the malic enzyme and alternative oxidase pathway probably function under conditions of relatively low ATP demands and high energy change characteristics at the later stages of the climacteric. While the regulation of electron transport via cytochrome and alternative pathways remains to be clarified in avocado mitochondria, the involvement of the alternative pathway cannot be totally ruled out.

b. Phosphofructokinase and Pyrophosphate: Fructose-6-Phosphate Phosphotransferase

Salimen and Young (1975) examined the possibility that the climacteric was regulated by enzyme activation involving phosphofructokinase (PFK) (ATP:D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11). This was based on research by Barker and Solomos (1962), who observed an increase in fructose 1,6-diphosphate during the ripening of bananas and in tomatoes by Chalmers and Rowan (1971). This increase in fructose 1,6-diphosphate was attributed to activation of PFK. Salimen and Young (1975) reported that activation of this enzyme accounted for a 20-fold increase in fructose 1,6-diphosphate during the ripening process. Electrophoretic separation of PFK showed that no new species of the enzyme were produced during the climacteric, with the enzyme remaining in the oligomeric form. Rhodes (1971) reported that PFK was present in the oligomeric weight form species in tomato fruits up to the climacteric phase while both the oligomeric and low-molecular-weight species were isolated in the postclimacteric phase. Isaac and Rhodes (1982) later found that PFK existed in the oligomeric form at the 'breaker' stage during tomato ripening. Using gel-permeation chromatography Isaac and Rhodes (1987) identified a single

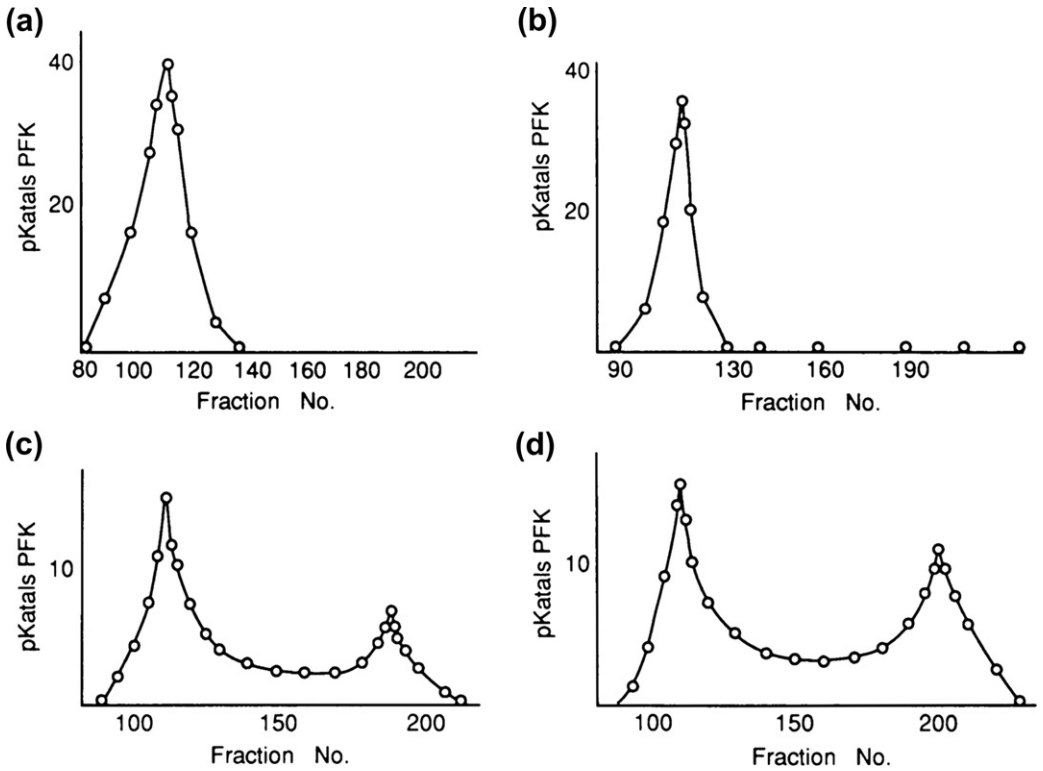


FIGURE 2.3 Elution of phosphofructokinase (PFK) on Ultrogel AcA 34 for the enzyme preparation from (a) green, (b) breaker, (c) orange, and (d) red tomatoes (Isaac and Rhodes, 1987). *(Reprinted with permission. Copyright © by Pergamon Press.)*

peak corresponding to the oligomeric form of PFK at the green and breaker stages. Two peaks were separated, however, at the orange and red stages of tomato ripening, which corresponded to oligomeric and monomeric forms of the enzyme (Figure 2.3). To explain the behavior of PFK, these researchers proposed that stimulation of the enzyme occurred because of leakage of inorganic phosphate (P_i) from the vacuole as a consequence of permeability changes in the membrane during initiation of the climacteric. The continued leakage of P_i and citrate affected the enzyme at the molecular level by dissociating the oligomeric form of the enzyme into monomeric subunits during the later stages of ripening.

A study by Bennett *et al.* (1987) examined the role of glycolytic regulation of the climacteric in avocado fruit. They used *in vivo* ^{31}P nuclear magnetic resonance spectroscopy to monitor the levels of phosphorylated nucleotides. They focused particular attention on pyrophosphate:fructose-6-phosphate phosphotransferase (PFP), an alternative enzyme identified in pineapple by Carnal and Black (1979). This enzyme catalyzes the identical reaction as PFK, utilizing PP_i instead of ATP as a phosphate donor, and is activated by fructose 2,6-biphosphate. An increase in the amount of fructose 2,6-phosphate concurrently with the rise in respiration suggested to these researchers that PFP may also be involved in the regulation of ripening in avocado fruit.

III. INITIATION OF RIPENING

Ethylene is one of many volatile substances emanating from fruits and vegetables which was subsequently identified by Gane (1934) as the active component for the stimulation of ripening. The application of minute quantities of ethylene, on the order of 1 ppm, stimulates respiratory activity, induces ripening, and hastens the onset of the climacteric. Thus, ethylene was soon recognized as a plant hormone that initiates the ripening process as well as regulating many aspects of plant growth, development, pathogen and wounding responses, senescence, and both abiotic and biotic stress responses (Abeles, 1973; Gazzarrini and McCourt, 2001; Alexander and Grierson, 2002). Based on respiration, fruits can be grouped as climacteric and non-climacteric, which was described in the previous part (Lelievre *et al.*, 1997). Ripening in climacteric fruits (Table 2.1) manifests itself by an upsurge in respiration and a concomitant burst of ethylene. In non-climacteric fruits respiration shows no change and ethylene production remains at a very low level during ripening. In addition to increased respiratory metabolism, ethylene stimulates its own biosynthesis in ripening climacteric fruits (Burg and Burg, 1965). The application of increased levels of ethylene to climacteric fruits hastens the onset of the climacteric rise, accompanied by an increase in oxygen uptake (Figure 2.4). Hence, ethylene is required for ripening because the ripening process can be inhibited by genetic control (Oeller *et al.*, 1991; Theologis *et al.*, 1993; Picton *et al.*, 1993; Ayub *et al.*, 1996; Brummell, 2005) or by ethylene action inhibitors such as 1-methyl-cyclopropene (1-MCP) (Sisler and Serek, 1997). With respect to non-climacteric fruits, an increase in oxygen absorption accompanies the application of ethylene. In the case of climacteric fruits, once ethylene exerts the respiratory rise the process cannot be reversed. This is in sharp contrast to non-climacteric fruits, in which the respiratory activity returns to the level of the control once ethylene treatment is terminated (Vendrell *et al.*, 2001).

At one time ethylene was considered a by-product rather than a ripening hormone as the amount present during the preclimacteric phase in many fruits was insufficient to stimulate ripening (Biale *et al.*, 1954). This conclusion was based on the amount of ethylene emanating from the fruit rather than the intracellular concentrations and was measured using manometric techniques that were far too insensitive. Subsequent research using gas chromatography provided ample evidence for the presence of ethylene in the intracellular spaces (Burg and Burg, 1965). It is generally accepted that the ethylene levels required to stimulate ripening fall within 0.1−1.0 ppm, completely beyond the range of normal manometric techniques. Table 2.2 summarizes the change in internal ethylene levels during the ripening of some climacteric and non-climacteric fruits. In the case of avocado, mango, and pears the ethylene level before the climacteric rise was lower than the accepted threshold of 0.1 ppm. Biale and Young (1971) noted that the rapid initiation of ripening of avocado required levels of ethylene greater than 1 ppm. These researchers pointed out some 10 years later (Biale and Young, 1981) that it was difficult to generalize about the minimum levels of ethylene needed to induce the climacteric rise because of the scant data available. Peacock (1972) suggested that the effectiveness of ethylene was a function of the log of its concentration, length of exposure, and time of application after harvest. As the fruit approached maturity it was evident that there was a decrease in sensitivity to ethylene.

However, studies by Vendrell and McGlasson (1971) on preclimacteric banana fruit tissue and sycamore figs (Zeroni *et al.*, 1976) reported that exogenous ethylene exerted a negative feedback regulation on ethylene production. Autoinhibition of ethylene production was also reported for non-climacteric fruit such as wounded

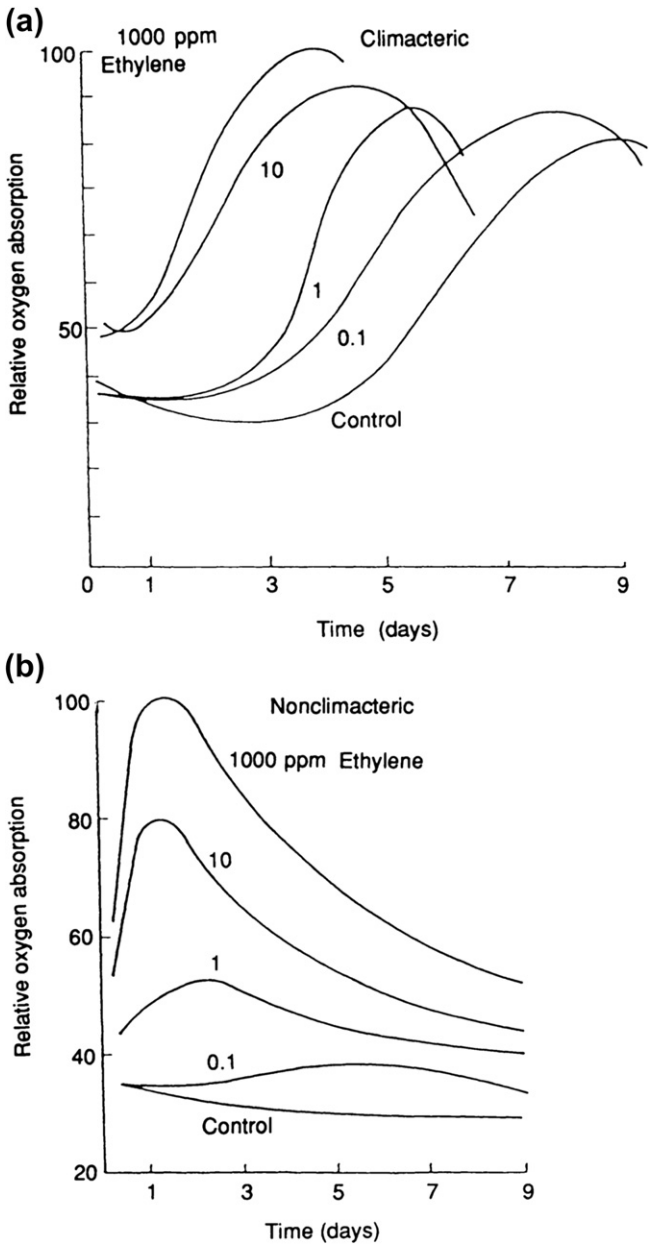


FIGURE 2.4 Oxygen uptake by fruits which show the climacteric phenomenon and by fruits which do not, in relation to concentration of external ethylene (Biale, 1964).

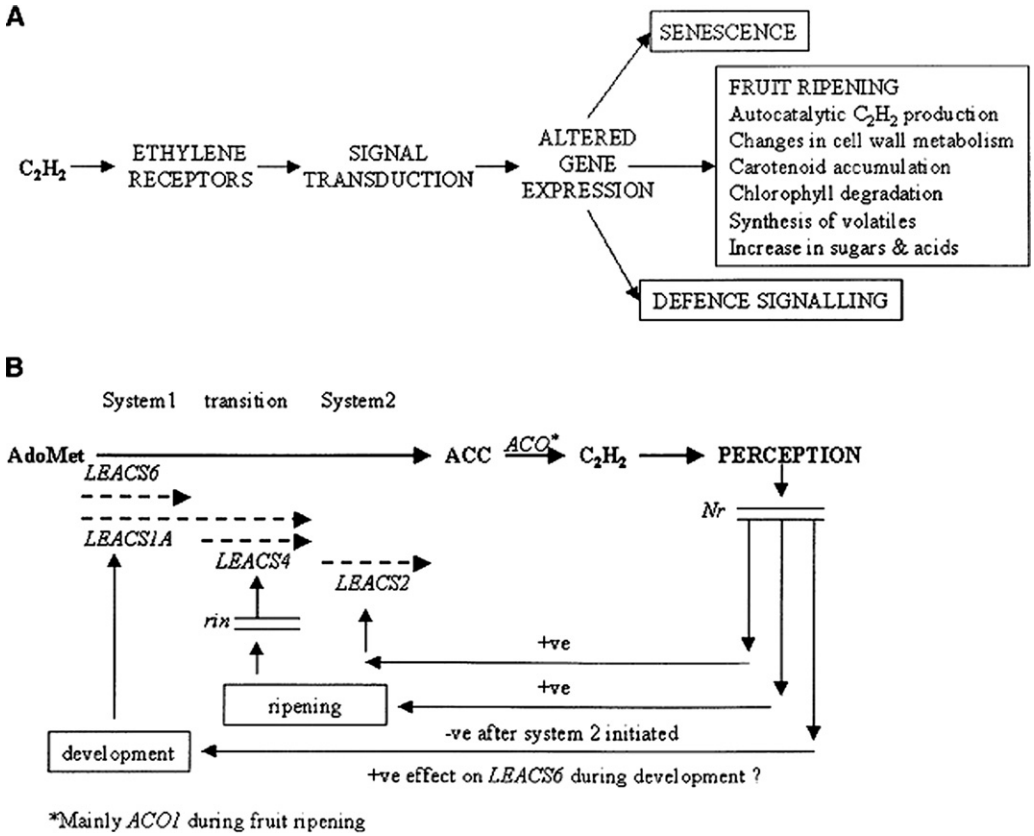
flavedo tissues of citrus (Riov and Yang, 1982a). On the basis of such observations, McMurchie *et al.* (1972) proposed the concept of system 1 and system 2 ethylene. System 1 is responsible for the low-rate production of ethylene in preclimacteric fruit and for most, if not all, ethylene produced by vegetative tissues. System 2 is accountable for the high-rate production of ethylene observed during the climacteric and is autocatalytically induced by ethylene. The transition to the system 2 is presumably due to the transcriptional activation of different ACC synthase (ACS) and ACC oxidase (ACO) genes (Scheme 2.2) (Barry *et al.*, 2000; Alexander and Grierson, 2002).

It is evident that ethylene plays a crucial and complex role in the regulation of climacteric fruit ripening. Ethylene affects encoding of many specific enzymes required for ripening, while some other compound(s), as yet unidentified, may induce this in non-climacteric fruits. Hence, it appears that both ethylene-dependent and -independent ripening

TABLE 2.2 Internal Ethylene Content (ppm) in some Climacteric and Non-climacteric Fruit

Fruit	Variety	Preclimacteric	Onset	Climacteric Peak
Climacteric				
Avocado[a]	Fuerte	0.03	0.09	25
Banana[b]	Gros Michel	0.1	1.5	40
Mango[b]	Kent Haden	0.01	0.08	3
Pear[c]	Anjou	0.09	0.4	40
Non-climacteric			Steady state	
Lemon[a]			0.1–0.2	
Orange[a]			0.1–0.2	
Lime[a]			0.3–2.0	

[a]*From Akamine and Goo (1979b)*
[b]*from Burg and Burg (1962)*
[c]*from Kosiyachinda and Young (1975)*



SCHEME 2.2 (A) Schematic representation of the role that ethylene plays in fruit ripening. (B) Model proposing the differential regulation of ACS gene during the transition from system 1 to system 2 ethylene synthesis. The symbols −ve (negative) and +ve (positive) refer to the action of ethylene on signaling pathways resulting in repression (−ve) or stimulation (+ve) of gene expression (Barry *et al.*, 2000; Alexander and Grierson, 2002).

pathways coexist in climacteric and non-climacteric fruits. Isolation of genes associated with the ripening processes in non-climacteric fruit may lead to progress in elucidating the regulation of fruit ripening (Aharoni *et al.*, 2000; Flores *et al.*, 2002; Li *et al.*, 2011).

IV. BIOSYNTHESIS OF ETHYLENE

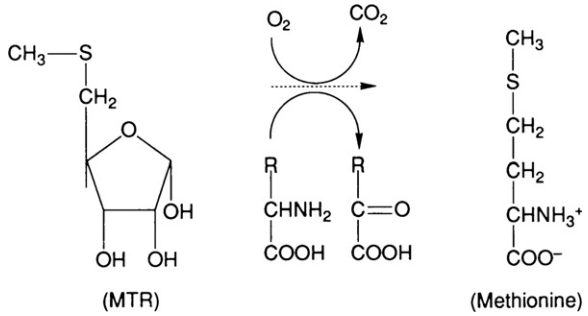
Various precursors of ethylene have been proposed but it is well established that methionine appears to be the main one in higher plants (Kende, 1993; Alexander and Grierson, 2002).

A. Methionine as a Precursor of Ethylene

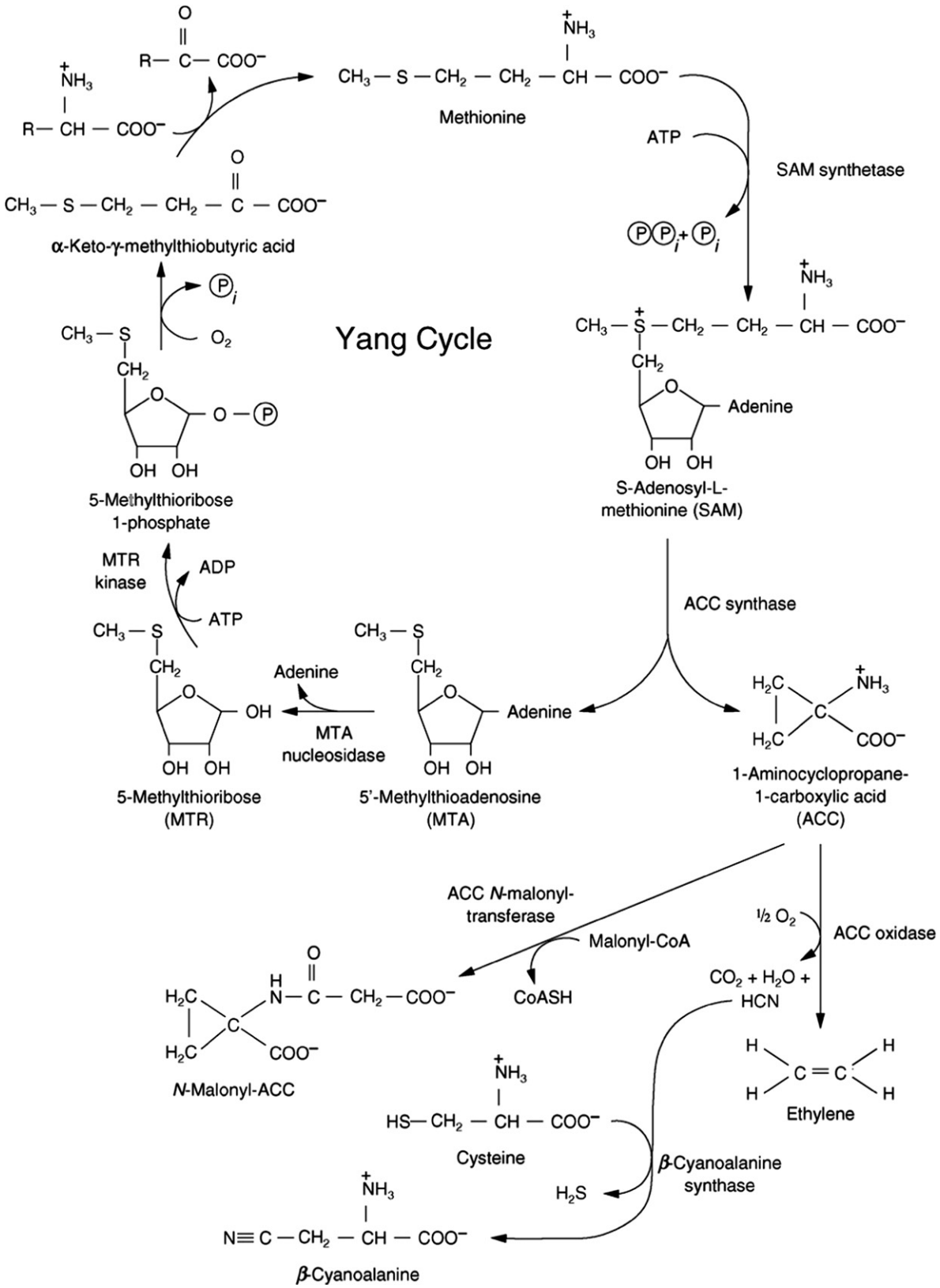
Lieberman and Mapson (1964) initially examined the production of hydrocarbons, including ethane and ethylene, in model systems containing peroxidized linoleic acid, Cu^{2+}, and ascorbic acid. To test whether ethylene production from linoleic acid involved free radicals they added a free radical quencher, methionine. Instead of methionine inhibiting the reaction they found that the production of ethylene was greatly enhanced. Further work showed that ethylene could be produced in the absence of peroxidized lipids as long as methionine−Cu^{2+}-ascorbate was present (Lieberman *et al.*, 1965). It was soon shown that methionine was in fact the biological precursor of ethylene in plants (Lieberman *et al.*, 1965; Yang, 1974). Using ^{14}C-labeled methionine, Lieberman *et al.* (1966) demonstrated its conversion to ethylene in apple fruit tissue. The fact that the C1 from methionine yielded carbon dioxide, and C3 and C4 yielded ethylene in both chemical systems and plant tissue, suggested that a common mechanism was involved. The two systems were quite different, however, as methionine was converted via methional with the methyl sulfide group yielding volatile dimethyl sulfide in the model systems. This differed in plant tissue, as methionine is limiting so that the sulfur group is recycled for resynthesis of methionine.

1. The Yang Cycle, Recycling of Methionine

The inhibition of ethylene production from methionine in the presence of DNP, an uncoupler of oxidative phosphorylation, suggested the formation of *S*-adenosyl-L-methionine (SAM) as an intermediate in this process (Burg, 1973; Murr and Yang, 1975). Using labeled methionine, Adams and Yang (1977) reported that the CH_3−S group of methionine was released as 5-methylthioadenosine (MTA) during ethylene synthesis in apple slices. MTA could only be formed as a degradation product if ethylene was synthesized from SAM. In addition to MTA, these researchers detected 5-methylthioribose (MTR), a degradation product of MTA in apple tissue. This suggested that the CH_3−S unit of MTR combined with a four-carbon receptor, such as homoserine, to form methionine, while the ribose group split off. It was subsequently found that the ribose unit of MTA/MTR was directly incorporated into methionine along with the CH_3−S group. Yung and Yang (1980) demonstrated that three MTR molecules were involved in methionine formation, with the ribose moiety modified to form the 2,3-aminobutyrate portion of methionine while the CH_3−S unit remained intact:



This pathway explains how methionine is recycled and maintained within plants. The overall pathway involved in the resynthesis of methionine from MTA is shown in Scheme 2.3. MTR-1 phosphate is converted to 2-oxo-4-methylthiobutanoic acid, from which methionine is re-formed. Miyazaki and Yang (1987) examined the methionine cycle enzymes in a number of fruits and showed that the conversion of MTR to methionine in ripening apples was not a limiting factor in the formation of ethylene. This cycle has been named the Yang cycle in plant biochemistry texts (Bradford, 2008).



SCHEME 2.3 The Yang cycle and formation of ethylene and other products from ACC (Bradford, 2008).

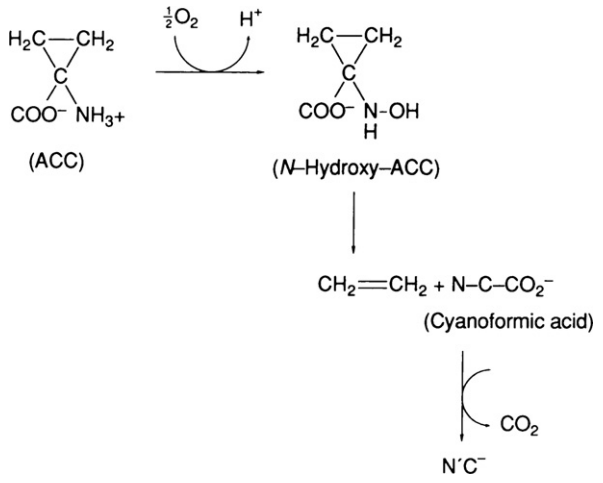
2. Methionine and Ethylene Biosynthesis

Early studies by Hansen (1942) and Burg and Thimann (1959) showed that ethylene production ceased when apples and pears were stored in an atmosphere of nitrogen. On re-exposure to oxygen, however, the production of ethylene was restored. The rapid production of ethylene suggested the accumulation of an intermediate compound during anaerobic storage. Adams and Yang (1979), using L[U-^{14}C]methionine, identified 1-aminocyclopropane-1-carboxylic acid (ACC) as the intermediate formed in apple fruit stored under nitrogen. It would appear therefore that methionine is first converted to *S*-adenosylmethionine, which then undergoes fragmentation to ACC and MTA. These researchers also found that labeled ACC was converted to ethylene when the apple tissue was incubated in air, which suggested the following sequence:

$$\text{Methionine} \rightarrow \text{SAM} \rightarrow \text{ACC} \rightarrow \text{Ethylene}$$

The conversion of methionine to SAM involves methionine adenosyl-transferase (ATP:methionine *S*-adenosyl-transferase, EC 2.5.1.6). This enzyme was reported in plant tissues by Konze and Kende (1979) in relation to ethylene production. The addition of aminoethoxylvinylglycine (AVG), an inhibitor of pyridoxal phosphate-mediated enzyme reactions (Rando, 1974), was subsequently shown to inhibit ethylene production from methionine. The part of the reaction sequence affected was SAM to ACC, which involved the participation of pyridoxal phosphate (Adams and Yang, 1979). The enzyme involved, ACC synthase, was identified in tomato preparations and shown to be activated by pyridoxal phosphate (Boller *et al.*, 1979; Yu *et al.*, 1979). ACC synthase was later identified and studied in apples (Bufler and Bangerth, 1983; Bufler, 1984), tomatoes (Acaster and Kende, 1983), cantaloupe (Hoffman and Yang, 1980), and citrus peel (Riov and Yang, 1982b).

The application of ACC to plant organs was shown by Lurssen *et al.* (1979) to enhance ethylene production. These researchers speculated that ACC was derived from methionine via SAM or ACC. The enzyme system involved in the formation of ethylene from ACC appeared to be associated with cellular particles (Mattoo and Lieberman, 1977; Imaseki and Watanabe, 1978). Disruption of the cellular membrane by either treatment with lipophilic compounds or osmotic shock reduced ethylene production in plant tissues (Odawara *et al.*, 1977; Imaseki and Watanabe, 1978). The particular step inhibited was identified as ACC to ethylene (Apelbaum *et al.*, 1981). An enzyme extract capable of converting ACC to ethylene was reported in pea seedlings by Konze and Kende (1979). Similar systems have been reported in a carnation microsomal system (Mayak *et al.*, 1981) and a pea microsomal system (McRae *et al.*, 1982). The search for the enzyme system that is responsible for the conversion of ACC to ethylene was finally comprehensively described by Kende (1993). It was long assumed that the ethylene-forming system was membrane associated and was referred to as the ethylene-forming enzyme (EFE). Yang and Hoffman (1984) suggested that ACC might be oxidized by an enzyme, ACC hydroxylase, to *N*-hydroxy-ACC, which is then broken down to ethylene and cyanoformic acid. The latter is extremely labile and spontaneously fragments to carbon dioxide and hydrogen cyanide (HCN). Support for this was based on studies by Peiser *et al.* (1983), who reported incorporation of [1-^{14}C] ACC into [4-^{14}C] asparagine in mung bean hypocotyls at levels similar to the production of ethylene. These findings, together with work by Miller and Conn (1980), who demonstrated incorporation of Na−CN into asparagine in mung bean, suggested the following pathway:



The identification of EFE was eventually accomplished based on molecular cloning of tomatoes (Slater *et al.*, 1983). The first ACO gene was discovered through antisense expression of a clone, pTOM13 (Holdsworth *et al.*, 1987). The role of this enzyme in ethylene synthesis was then confirmed by expression of pTOM13 in yeast and *Xenopus* oocytes (Hamilton *et al.*, 1990; Spanu *et al.*, 1991). Further ACO genes have been identified in tomato and other climacteric fruit such as apples, avocado, bananas, and melons (Holdsworth *et al.*, 1988; Barry *et al.*, 1996; Blume and Grierson, 1997; Nakatsuka *et al.*, 1998; Jiang and Fu, 2000; Llop-Tous *et al.*, 2000). Ververidis and John (1991) extracted EFE activity from melon fruits under N_2 gas and addition of Fe^{2+} and ascorbate. These conditions enabled full recovery of EFE activity and upon fractionation by centrifugation, the enzyme was recovered in the soluble fraction. Ververidis and John (1991) proposed that EFE be referred to as ACC oxidase (ACO) since it seemed to be related to 2-oxoglutarate-dependent dioxygenases which require Fe^{2+} and ascorbate for *in vitro* activity. ACO has since been extracted from apple (Dong *et al.*, 1992; Fernandes-Maculet and Yang, 1992; Kuai and Dilley, 1992) and from avocado (McGarvey and Christoffersen, 1992). Dong *et al.* (1992) stated that ACO activity was enhanced *in vivo* by carbon dioxide and *in vitro* it was entirely dependent on the presence of carbon dioxide. Furthermore, they observed that ACO exhibited an absolute requirement for Fe^{2+} and ascorbate but not for 2-oxoglutarate. Based on these observations they postulated the following stoichiometry for the conversion of ACC to ethylene:

$$\text{ACC} + \text{O}_2 + \text{ascorbate} \xrightarrow{\text{Fe}^{2+},\ \text{CO}_2} \text{C}_2\text{H}_4 + \text{CO}_2 + \text{HCN} + \text{dehydroascorbate} + 2\text{H}_2\text{O}$$

The role of the Fe(II) ion is to bind ACC and O_2 simultaneously and promote electron transfer, which initiates catalysis of ACC to ethylene (Pirrung, 1999; Rocklin *et al.*, 1999).

B. Regulation of Ethylene in Ripening Fruits

1. ACC Synthase and ACC Oxidase

The climacteric rise in fruits is associated with enhanced ethylene production at the onset of ripening. Positive feedback regulation of ethylene biosynthesis is a characteristic feature of ripening fruits. Exposure to exogenous ethylene triggers a large increase in ethylene production due to the induction of both key enzymes, ACS and ACO (Chang and Bleecker, 2004; Génard and Gouble, 2005; Li *et al.*, 2011). The changes in the internal level of ACC were examined by Hoffman and Yang (1980) during the ripening of avocados as well as the effect of exogenous ACC on ethylene synthesis in the preclimacteric fruit. Their results in Figure 2.5 show that ACC was present at extremely low levels in the preclimacteric fruit (< 0.1 nmole/g) but increased dramatically just before the onset of ethylene, decreasing to 5 nmole/g in the overripe fruit. The low level of ACC in the preclimacteric fruit was attributed to the inability to convert SAM to ACC. Addition of exogenous ACC to the preclimacteric tissue did increase ethylene production but only to a limited degree (Adams and Yang, 1977, 1979). Thus, the formation of ACC from SAM appeared to be the rate-controlling step in the biosynthesis of ethylene (Yang, 1980). Further confirmation was provided by Liu *et al.* (1985), who studied the effect of ethylene treatment on the production of ethylene in climacteric tomato and cantaloupe fruits. They found that when exposed to exogenous ethylene the increased activity of the ethylene-forming enzyme (ACO) preceded any increase in ACC synthase in these preclimacteric fruits. Examination of *ACO*mRNA expression patterns in different tissues and at different developmental stages confirmed the regulatory role of ACO in the ethylene production during ripening (Holdsworth *et al.*, 1987; Hamilton *et al.*, 1990; Balague *et al.*, 1993; Barry *et al.*, 1996; Alexander and Grierson, 2002).

A variety of stresses, such as wounding, hypoxia, chilling, freezing, or drought, can induce ethylene biosynthesis as a result of the increase in ACC synthase activity (Wang *et al.*, 2002). Morin *et al.* (1985) found that cold storage of 'Passé-Crassane' pears was required to initiate ethylene ripening and induce the synthesis of free or conjugated ACC. During cold storage (0°C) both free and conjugated ACC increased together with ribosomes and mRNA. When these pears were then transferred to 15°C, a marked rise in ethylene occurred, followed by the climacteric period (Hartmann *et al.*, 1987).

2. Cyanide

The production of cyanide in the biosynthesis of ethylene from ACC was shown in studies by Peiser *et al.* (1984) and Pirrung (1985). Pirrung and Brauman (1987) suggested that cyanide might regulate ethylene formation during the climacteric period. They proposed that in ethylene biosynthesis the cytochrome and cyanide-resistant respiratory chains were connected via cytochrome *c* oxidase. The inhibition of cytochrome *c* oxidase by cyanide during ethylene

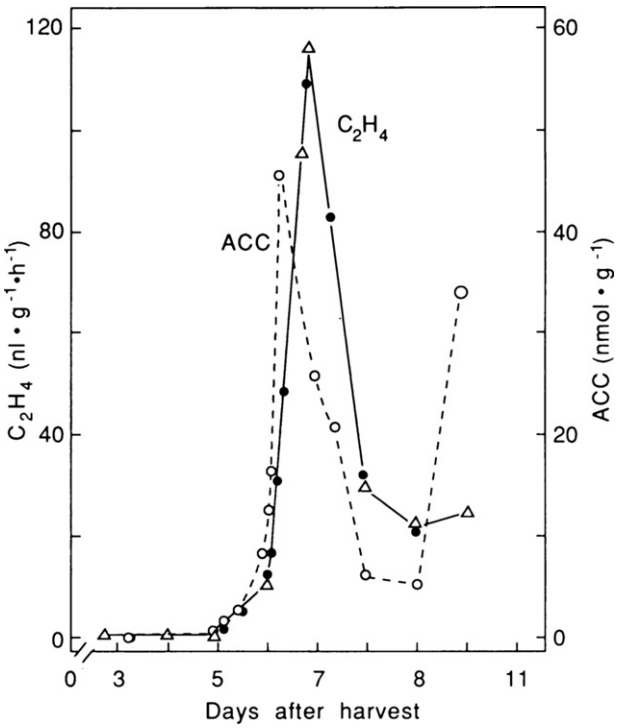


FIGURE 2.5 Changes in ACC content of avocado fruit at various stages of ripening. Each ACC value is from a single fruit which has been monitored for ethylene production and assigned an arbitrary stage of ripeness by comparison with the established climacteric patterns of ethylene production (Hoffman and Yang, 1980).

biosynthesis favored the alternative pathway which, in turn, led to ACC synthesis. Gene expression for ACC synthase in response to the alternative respiratory pathway could explain differences between climacteric and non-climacteric fruit. However, there is evidence that plant tissues possess ample capacity to detoxify HCN formed during ethylene biosynthesis and that the concentration of HCN is kept at a low level (Yip and Yang, 1988). The key enzyme to detoxify HCN is β-cyanoalanine synthease (EC 4.4.1.9). The β-cyanoalanine is further metabolized to asparagine or to γ-glutamyl-β-cyanoalanine.

3. Organic Acids

De Pooter *et al.* (1982) observed an increase in carbon dioxide production and premature ripening when intact 'Golden Delicious' apples were treated with propionic and butyric acids. This change was identical to apples treated with ethylene and suggested a role for these acids in ethylene production. It appeared feasible that ethylene could be produced from these carboxylic acids according to system 1, which then triggered normal ethylene production via system 2. Further work by these researchers (De Pooter *et al.*, 1984) confirmed the premature ripening of the intact 'Golden Delicious' apples when treated with acetic or propionic acid vapors. A small part of labeled [2-^{14}C]propionic acid was transformed into [^{14}C]ethylene which then acted as a trigger for ripening (system 2). The degree of fruit maturity was thought to be a major factor, which suggested that in the unripe apples the small amount of ethylene produced was probably derived from simple organic acids. Thus, the concentration at which ethylene can trigger ripening depends on the availability of simple organic acids. The ability of carbon dioxide to delay the onset of ripening in fruits was shown by Bufler (1984) to be due, in part, to inhibition of ACC synthase development (Figure 2.6).

4. Lipid Peroxidation: Lipoxygenase

The production of ethylene has been correlated with changes in hydroperoxide levels, peroxidase activity, as well as increases in lipoxygenase activity in ripening fruits (Meigh *et al.*, 1967; Frenkel and Eskin, 1977; Frenkel, 1979; Marcelle, 1991; Sheng *et al.*, 2000; Liu *et al.*, 2008). Studies by Adams and Yang (1979) and Konze *et al.* (1980) both

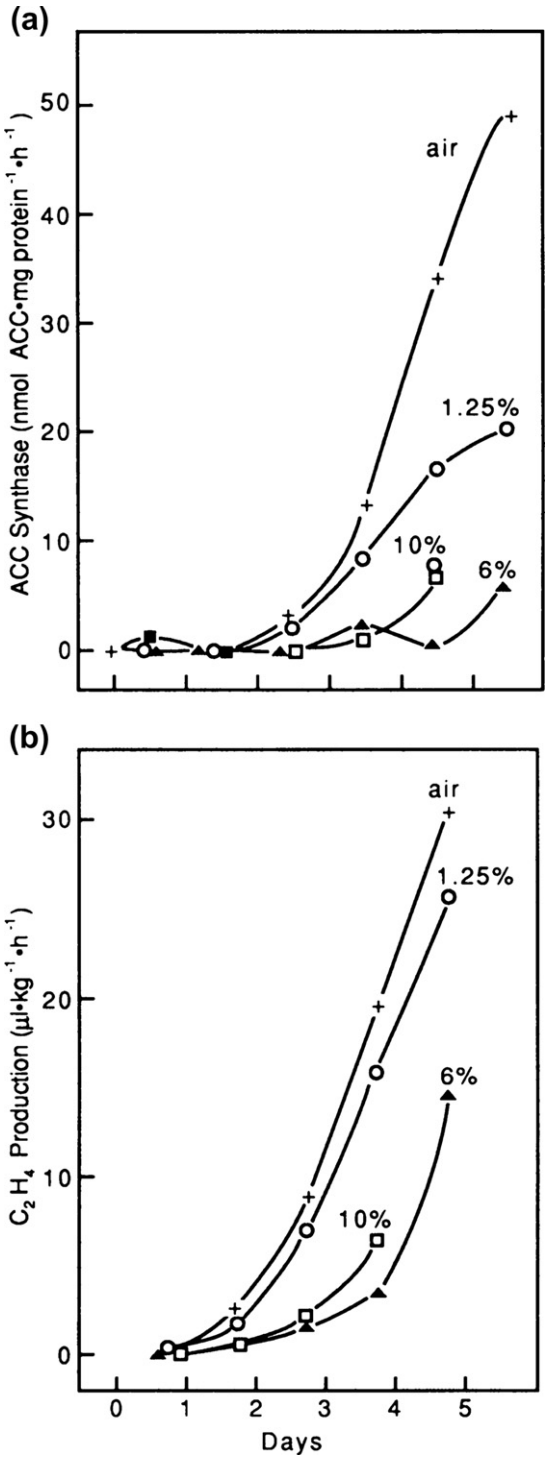


FIGURE 2.6 Effect of different concentrations of CO_2 on induction and development of ACC synthase activity (a) and ethylene production (b) in preclimacteric treated apples (Bufler, 1984). Apples were transferred from hypobaric storage to normal pressure and 25°C and immediately treated with air (+), 1.25% (○), 6% (▲), or 10% (□) CO_2 (Bufler, 1984). *(Reprinted with permission of copyright owner, American Society of Plant Physiology (ASPP)).*

suggested peroxidation as one mechanism for the formation of ethylene from ACC; however, the involvement of peroxidase was discounted by later researchers (Machackova and Zmrhal, 1981; Rohwer and Mader, 1981). The enzymatic conversion of ACC to ethylene in a cell-free system was found to be sensitive to catalase and inhibited by hydrogen peroxide (Konze and Kende, 1979; McRae *et al.*, 1982). The effect of hydroperoxides on the enzymatic

conversion of ACC to ethylene was investigated by Legge and Thompson (1983) using a model system composed of microsomal membranes from etiolated peas. Addition of hydroperoxides stimulated ethylene production in model systems containing ACC, as shown in Figure 2.7. Hydrogen peroxide, a known inhibitor of lipoxygenase, was found to inhibit ethylene formation. Lipoxygenase forms lipid hydroperoxides from linoleic acid (Eskin *et al.*, 1977). A 1.5-fold increase in ethylene production occurred following the addition of linoleic acid to model systems containing this enzyme. There appeared to be an interaction between lipoxygenase activity, a hydroperoxide derivative, and the ethylene-forming enzyme (ACO). McRae *et al.* (1982) provided evidence, based on spin-trapping data, that oxygen was involved in the formation of ethylene from ACC by pea microsomal membranes. Since hydroperoxides facilitate oxygen activation, the promotion of oxygen via this mechanism could lead to the formation of ethylene. Legge *et al.* (1982) detected free radical formation using a diagnostic spin trap 4-MePyBN which required ACC, oxygen, and hydroperoxides. Their results suggested that free radicals were derived from ACC in the microsomal system producing ethylene. Conversion of ACC to ethylene by pea microsomal membranes is mediated via a free radical intermediate requiring hydroperoxides and oxygen. The increase in free radical formation was attributed by Kacperska and Kubacka-Zabalska (1984) to lipoxygenase-mediated oxidation of polyunsaturated fatty acids. This was confirmed with *in vitro* and *in vivo* studies by Kacperska and Kubacka-Zabalska (1985), who found that an increase in ethylene from ACC in winter rape leaf disks resulted from lipoxygenase activity. Similar observations were reported by Bousquet and Thimann (1984) using oat leaf segments. In more recent studies relationships between lipoxygenase (LOX) activity and ethylene biosynthesis have been reported for kiwi fruit (Xu *et al.*, 2003; Zhang *et al.*, 2006), tomato (Sheng *et al.*, 2000), and oriental pear cultivars (Xu *et al.*, 2008). However, LOX

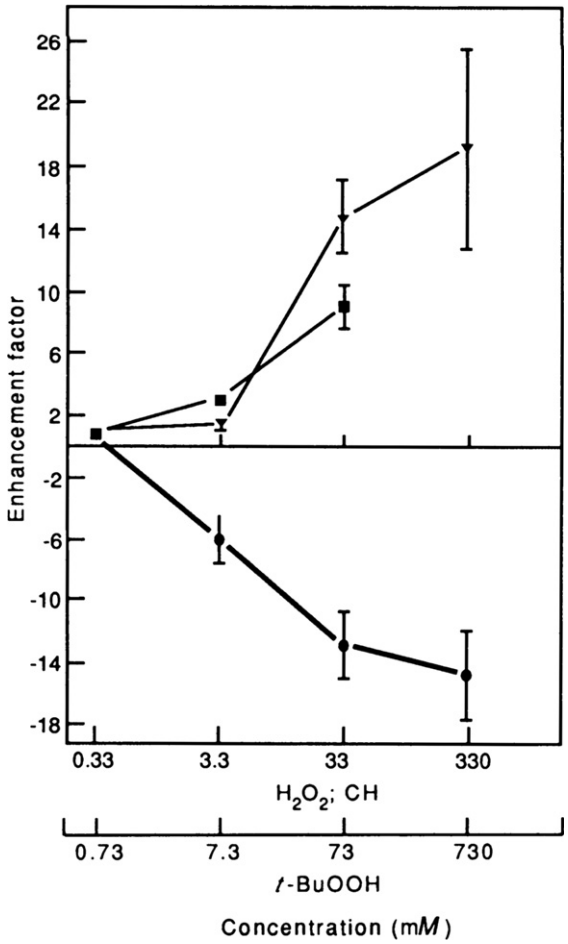


FIGURE 2.7 Effects of hydrogen peroxide (●), *t*-butylhydroperoxide (▲), and cumene hydroperoxide (■) on the conversion of ACC to ethylene by pea microsomal membranes. Final concentrations of hydroperoxide added to the basic reaction mixture are indicated along the abscissa. The enhancement factor is the ratio of ethylene produced in the presence of added hydroperoxide relative to that produced in its absence. Values represent the mean ± SE ($n = 3$) (Legge and Thompson, 1983). *(Reprinted with permission. Copyright © by Pergamon Press.)*

activity may be involved in ethylene biosynthesis as well as several aspects of fruit ripening affecting flavor development and response to low temperatures.

5. Galactose

Studies by Gross (1985) and later by Kim *et al.* (1987) showed that ethylene production was stimulated by galactose during the ripening of tomato fruit. Galactose is a product of reduced cell wall synthesis of galactan and increased activity of cell wall galactosyl residues by β-galactosidase (Lackey *et al.*, 1980; Pressey, 1983). Stimulation of ACC synthase activity by exogenous galactose to the pericarp tissue of green tomatoes suggested a relationship between cell wall turnover and ethylene biosynthesis in ripening tomato fruit (Kim *et al.*, 1987, 1991). Other studies confirmed that cell wall fragments stimulated or induced ethylene production (Tong *et al.*, 1986; Brecht and Huber, 1988). Later studies indicated relationships between short-chain oligogalacturonides and induction and expression of a gene encoding ACO in tomato plants and the regulation mode of transcription factors (Simpson *et al.*, 1998; Li *et al.*, 2011). Similarly, relationships between PG gene expression and softening and ethylene production were reported for kiwi fruit (Wang *et al.*, 2000).

V. COLOR CHANGES

One of the first changes observed during the ripening of many fruits is the loss of green color. This is followed by the development of red colors in some fruits and vegetables due to the formation of anthocyanins. The color changes in some fruits are summarized in Table 2.3. These changes take place immediately following the climacteric rise in respiration and are accompanied by textural changes in the fruit. In the case of leafy vegetables such as cabbage, lettuce, and Brussels sprouts, the loss of chlorophyll is also responsible for the symptom of yellowing during senescence (Lipton, 1987; Lipton and Ryder, 1989).

A. Chlorophyll Changes during Ripening

Ethylene has been reported to promote the degradation of chlorophyll during fruit ripening (Burg and Burg, 1965). A study by Hardy *et al.* (1971) noted the stimulation of chlorophyll biosynthesis in excised cotyledons of cucumber seeds (*Cucumis sativus*) by ethylene and light. Further work by Alscher and Castelfranco (1972) found that stimulation of chlorophyll synthesis occurred only in the dark as exposure to light inhibited chlorophyll synthesis. Little reference can be found on the stimulation of chlorophyll synthesis by ethylene, although the cucumber has provided an excellent system for studying chlorophyll production over the past decade (Pardo *et al.*, 1980; Chereskin *et al.*, 1982; Fuesler *et al.*, 1982; Hanamoto and Castelfranco, 1983). In contrast to stimulation of chlorophyll synthesis by ethylene in the dark, the biosynthesis of anthocyanins in red cabbage was only stimulated by ethylene when exposed to light.

B. Chlorophyll Biosynthesis

Chlorophylls, the major plant tetrapyrroles, are essential to human and animal life via their role in photosynthesis. The biosynthesis of chlorophyll is a highly coordinated process that involves numerous enzymes (Beale, 1999). By applying molecular genetic techniques it has been possible to identify and characterize those genes encoding

TABLE 2.3 Color Changes Occurring in Some Fruits During Ripening

	Ripening Stage	
Fruit	**Immature**	**Ripe**
Apple	Green	Yellow/red[a]
Banana	Green	Yellow
Pear	Green	Yellow
Strawberry	Green	Red

[a]*Depending on which variety*

TABLE 2.4 Genes Encoding the Enzymes in the Chlorophyll Biosynthetic Pathway in Angiosperms (Beale, 2005)

Step[a]	Enzyme Name	Gene Name(s)[b]
1	Glutamyl-tRNA reductase	*HEMA1*
		HEMA2
		HEMA3
2	Glutamate 1-semialdehyde aminotransferase (glutamate 1-semialdehyde aminomutase)	*GSA1* (*HEM1*)
		GSA2 (*HEM2*)
3	Porphobilinogen synthase (5-aminolevulinate dehydratase)	*HEMB1*
		HEMB2
4	Hydroxymethylbilane synthase (porphobilinogen deaminase)	*HEMC*
5	Uroporphyrinogen III synthase (uroporphyrinogen III co-synthase)	*HEMD*
6	Uroporphyrinogen decarboxylase	*HEME1*
		HEME2
7	Coproporphyrinogen oxidative decarboxylase	*HEMF1*
		HEMF2
8	Protoporphyrinogen oxidase	*HEMG1*
		HEMG2
9	Mg chelatase D subunit	*CHLD*
	Mg chelatase H subunit	*CHLH*
	Mg chelatase I subunit	*CHLI1*
		CHLI2
10	Mg-protoporphyrinogen IX methyltransferase	*CHLM*
11	Mg-protoporphyrinogen IX monomethylester cyclase	*CRD1* (*ACSF*)
12	Divinyl reductase	*DVR*
13	NADPH:protochlorophyllide oxidoreductase	*PORA*
		PORB
		PORC
14	Chlorophyll synthase	*CHLG*
15	Chlorophyllide *a* oxygenase	*CAO* (*CHL*)

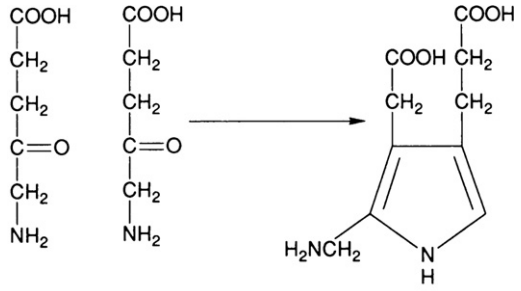
[a] *The step numbers correspond to the numbers in Scheme 2.4.*
[b] *The gene names are those for* Arabidopsis thaliana. *Alternative names for the enzymes and genes are indicated in parentheses. Multiple genes for a given enzyme are indicated by numerical suffixes, except for POR genes (step 13), for which letter suffixes are used.*

these enzymes (Beale, 2005) (Table 2.4). The biosynthesis of chlorophyll appears to involve three distinct phases (Tanaka and Tanaka, 2006). The first phase is the biosynthesis of chlorophyll *a* from glutamate (Vavilin and Vermass, 2002; Willows, 2003; Eckhardt and Grimm, 2004; Grossman *et al.*, 2004). The second phase, also known as the chlorophyll cycle, involves the interconversion of chlorophyll *a* to chlorophyll *b* (Ruediger, 2002). The third and final phase of chlorophyll metabolism concerns itself with degradation of chlorophyll *a* (Pinta *et al.*, 2000; Eckhardt and Grimm, 2004).

1. Phase 1: Glutamate to Chlorophyll a

Shemin and Russell (1953) first demonstrated the role of δ-aminolevulinic acid (ALA) in the biosynthesis of the tetrapyrrole nucleus of chlorophyll. The biosynthesis of ALA involves two distinct pathways (von Wettstein *et al.*, 1995). One pathway, used by animals (for heme), yeasts, and a number of bacteria, involves condensation of succinyl-CoA and glycine by the pyridoxal-P-containing enzyme 5-aminolevulinic acid synthase (EC 2.3.1.37) (Gibson *et al.*, 1958; Kikuchi *et al.*, 1958). The second and characteristic pathway in higher plants and algae, known as the C_5 pathway, is a three-step pathway in which glutamate is subsequently converted to 5-aminolevulinate. Using ^{14}C-labeled glutamate the intact five-carbon skeleton was shown to be directly incorporated into 5-aminolevulinate (Beale *et al.*, 1975; Meller *et al.*, 1975; Porra, 1986). The conversion to ALA requires activation of glutamate at the α-carbonyl by ligation to $tRNA^{Glu}$ (Kannangara *et al.*, 1984). ALA formation, the first step in the biosynthesis of chlorophyll, was thought to regulate the total amount of tetrapyrroles formed (Beale, 1999). Subsequent research suggested that ALA synthesis is subjected to feedback regulation by heme, and inhibited by FU, a regulatory protein, in the dark (Meskauskiene *et al.*, 2001; Goslings *et al.*, 2004). The initial reactions responsible for the synthesis of ALA in higher plants are shown in Scheme 2.4.

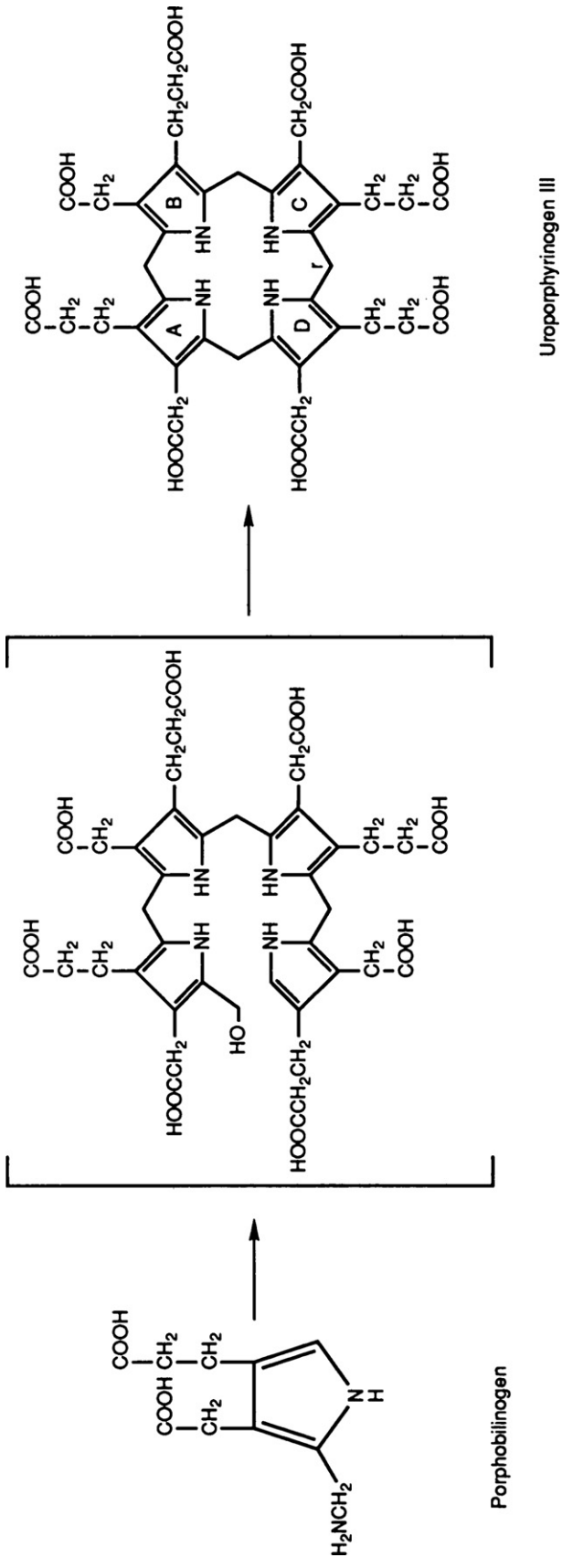
Activation of glutamate to glutamyl tRNA by glutamyl-tRNA synthetase (EC 6.1.1.17) involves ligation of $tRNA^{Glu}$, a process normally associated with plastid protein synthesis. The activated carboxyl group of glutamyl tRNA is then reduced to a formyl group by glutamyl-tRNA reductase (GluTR), resulting in the formation of glutamate-1-semialdehyde (GSA). This is followed by intermolecular amino-exchange reactions in which GSA is converted to ALA (Tanaka and Tanaka, 2007). Glutamate 1-semialdehyde aminotransferase (EC 5.4.3.8) catalyzes this reaction with pyridoxal-P or pyridoxamine-P as cofactor. The mechanism appears to involve the catalysis of two successive transformation reactions (Mau and Wang, 1988; Mayer *et al.*, 1993). A diamino intermediate, 4,5-diaminovaleric acid, is initially formed by transfer of an amino group from pyridoxamine-P to GSA, forming pyridoxal-P. In the second reaction, the amino group is transferred back to pyridoxal-P, regenerating pyridoxamine-P and forming ALA. This is supported by kinetic studies based on time-resolved spectrophometric measurements (Smith *et al.*, 1998). Once ALA is formed two molecules condense to form porphobilinogen (PBG), catalyzed by ALA dehydratase [5-aminolevulinate hydrolase (ALAD), EC 4.2.1.24] (Dresel and Falk, 1953; Schmid and Shemin, 1955). It is during this step that an aliphatic compound is converted into an aromatic one.



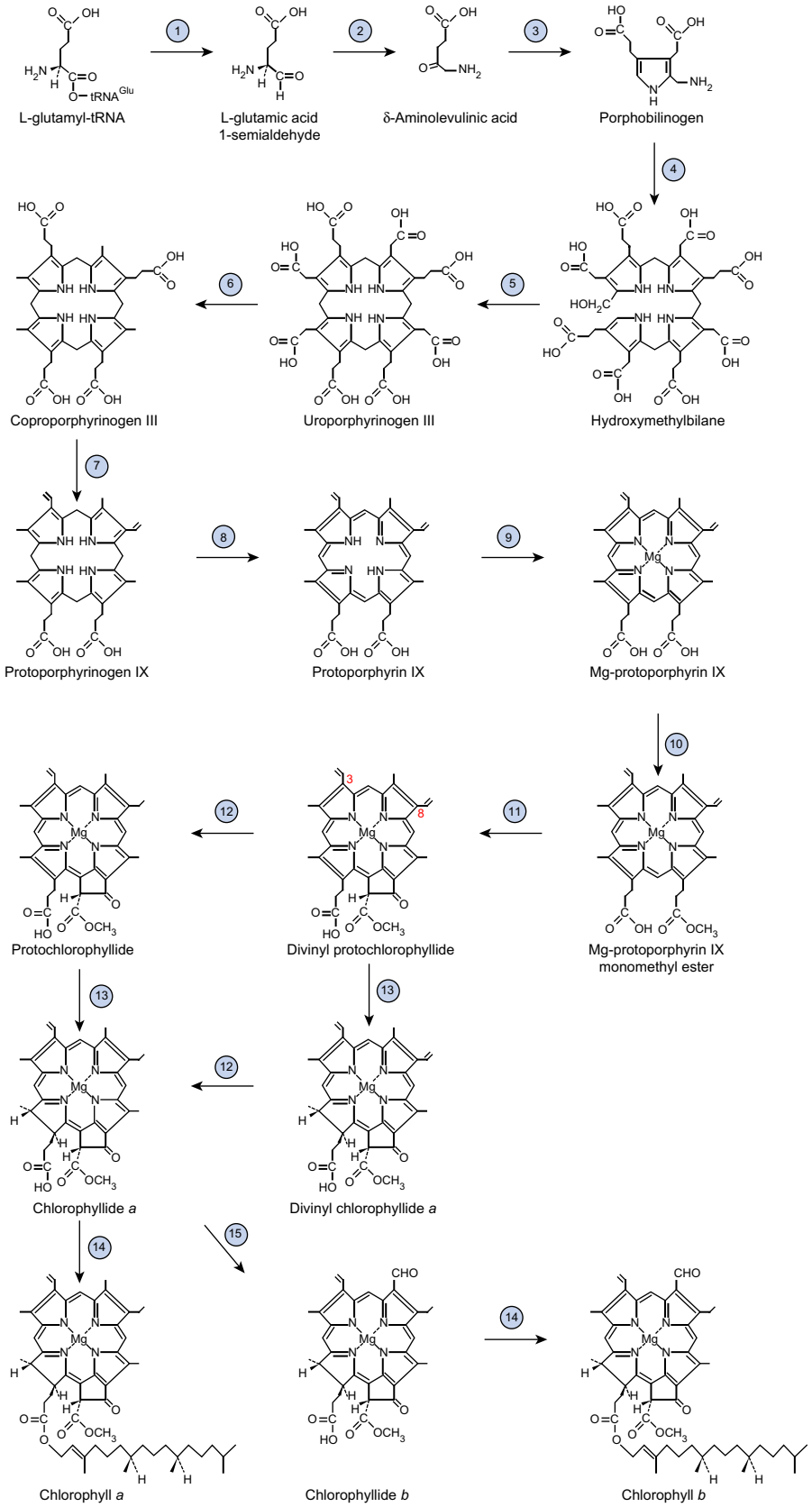
(2) 5–Aminolevulinic acid Porphobilinogen

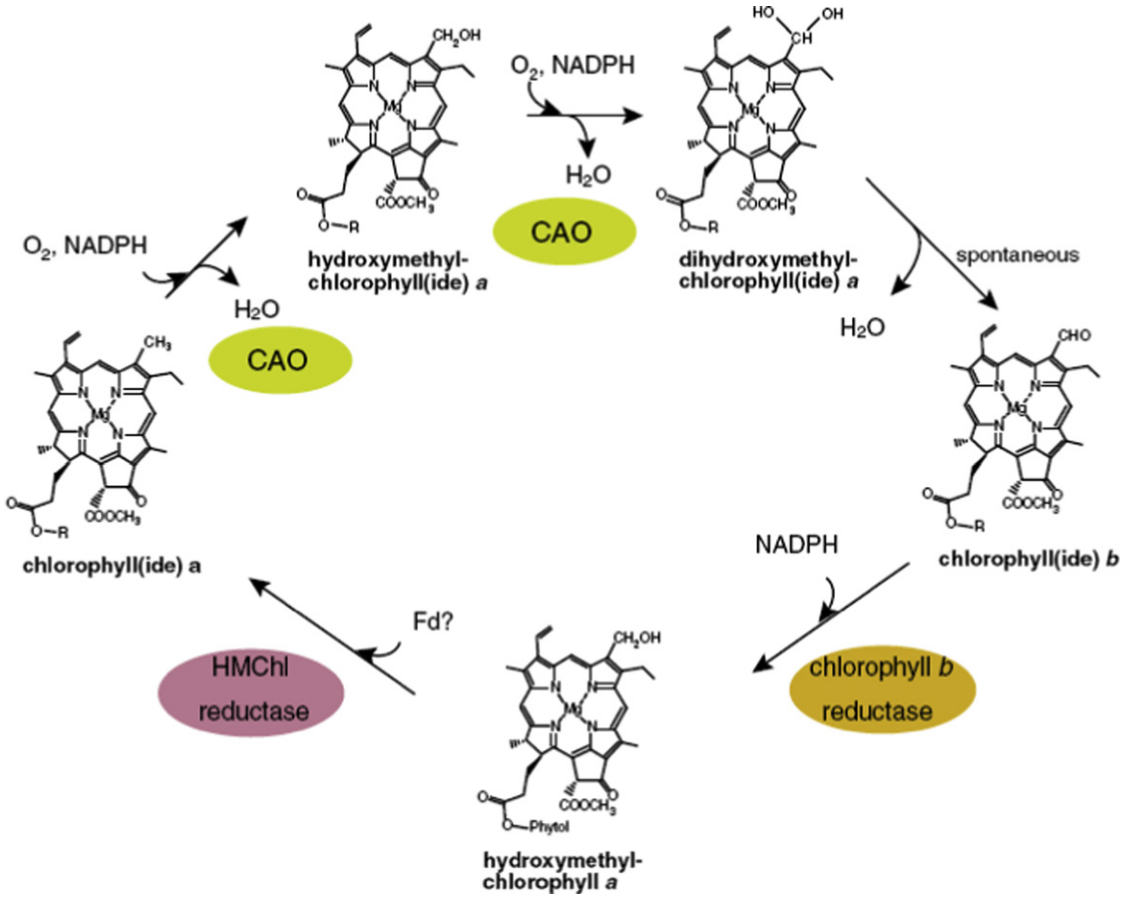
ALAD has been studied extensively in animal tissue and photosynthetic bacteria and in a few plants including wheat (Nandi and Waygood, 1967), soybean tissue culture (Tigier *et al.*, 1968, 1970), mung bean (Prasad and Prasad, 1987), and spinach (Liedgens *et al.*, 1983). ALAD resides in the chloroplasts, where it appears in the soluble form in the plastid stroma or loosely bound to lamellae.

The first tetrapyrrole intermediate, a linear hydroxymethylbilane porphyrin precursor, was identified by Battersby *et al.* (1979) and Jordan and Seehra (1979). This results from the head-to-tail condensation of four molecules of PBG catalyzed by PBG deaminase. This linear molecule is enzymatically closed by uroporphyrinogen III synthase to form the first cyclic tetrapyrrole, uroporphyrinogen III.


Porphobilinogen
Uroporphyrinogen III

SCHEME 2.4 Biosynthesis of chlorophyll in higher plants. Numbered arrows refer to the enzymes listed in Table 2.4. Reactions 12 and 13 can occur in either order, depending on the availability of substrates. Reaction 14 can use either of the substrates indicated. The position numbers of the two vinyl groups are indicated for 3.8-divinyl protochlorophyllide (Beale, 2005).





SCHEME 2.5 Proposed biosynthetic route of the chlorophyll cycle. The forward reaction may occur with chlorophyll or chlorophyllide. The chlorophyll to chlorophyllide conversion and reverse reaction are catalyzed by chlorophyllase and chlorophyll synthase, respectively, neither of which are depicted in the diagram. R = either proton or phytol; Fd = ferrdoxin (Tanaka *et al.*, 2011).

The steps leading to the formation of protoporphyrin IX, outlined in Scheme 2.5, will only be discussed briefly, as a detailed discussion can be found in several excellent reviews (Avissar and Moberg, 1995; Jaffe, 2003, 2004; Shoolingin-Jordan, 2003; Beale, 2005). Uroporphyrinogen III is converted to coproporphyrinogen III by uroporphyrinogen III decarboxylase, which decarboxylates the acetic acid groups on the pyrrole rings A, B, C, and D (Jackson *et al.*, 1976). This is followed by oxidative decarboxylation of the propionic acid groups on pyrrole groups A and B by the oxygen-dependent co-proporphyrinogen III oxidase (CPOX), forming protoporphyrinogen IX (Games *et al.*, 1976). The final step is the formation of protoporphyrin, during which six electrons are removed from protoporphyrinogen IX by the FAD-containing enzyme protoporphyrinogen IX oxidase (Poulson and Polglase, 1975).

Chelation of protoporphyrin IX is mediated by Mg chelatase and requires a high concentration of ATP (Pardo *et al.*, 1980). This enzyme is composed of three subunits, Ch1H, Ch1I, and ChID, with average molecular weights of 140, 40, and 7 kDa, respectively. The catalytic site is on Ch1H, which is activated by Ch1I and Ch1D binding together (Davison *et al.*, 2005). The enzyme, Mg-protoporphyrin IX methyltransferase (MgMT), then transfers a methyl group from *S*-adenosyl-L-methionine to the carboxyl group on 13-proprionate to form Mg-protoporphyrin-*N*-monomethyl ester (Fuesler *et al.*, 1982). Fuesler and co-workers (1982) demonstrated the following reaction sequence in which metal chelation preceded methylation. Using a high-performance liquid chromatography (HPLC) procedure to separate Mg-protoporphyrin and Mg-protoporphyrin-Me ester, Fuesler *et al.* (1982) demonstrated the following reaction sequence:

$$\text{Protoporphyrin IX} \xrightarrow{Mg^{2+}ATP} \text{Mg-protoporphyrin} \xrightarrow{SAM} \text{Mg-protoporphyrin-Me}$$

Mg-protoporphyrin-Me ester is then converted by Mg-protoporphyrin IX monomethyl cyclase (MgCy) to 3,8-divinyl-protochlorophyllide by incorporating an atomic oxygen. Protochlorophyllide oxidoreductase (POR) then reduces the D ring of 3,8-divinyl protochlorophyllide to 3,8-divinyl chlorophyllide. The 8-vinyl substituent in the side-chain of the pyrrole ring B is then reduced by divinylchlorophyllide reductase (DVR) to 3-vinyl chlorophyllide *a* (monovinyl chlorophyllide *a*). DVR can also reduce 3,8-divinyl protochlorophyllide but the efficiency of this substrate was reported by Tanaka and Tanaka (2007) to be substantially lower than 3,8-divinyl chlorophyllide. Based on this, they revised the conventional reaction steps by placing the DVR reaction after the POR reaction. The final step is the biosynthesis of chlorophyll *a* from 3-vinyl chlorophyllide. Chlorophyll synthase esterifies the 17-proprionate substituent on pyrrole ring D of monovinyl chlorophyllide *a* with geranyl geraniol, which is reduced to phytol.

2. Phase 2: The Chlorophyll Cycle

In this phase a portion of the chlorophyll *a* pool is converted to chlorophyll *b* (Scheme 2.5). This is a two-step process catalyzed by a single polypeptide. The enzyme involved, a Rieske-type monooxygenase, chlorophyllide *a* oxygenase (CAO), was first characterized by Tanaka and co-workers (Tanaka *et al.*, 1998; Espineda *et al.*, 1999). Cloning and functional expression of the CAO gene subsequently showed that CAO catalyzed two oxygenation steps in which chlorophyllide *a* was converted to chlorophyllide *b* (Oster *et al.*, 2000). The first oxygenation step by CAO produced the intermediate 7-hydroxymethylchlorophyllide *a*, which was then oxygenated to chlorophyllide *b*. The latter is then phytylated by chlorophyll synthase to chlorophyll *b*. A recent review by Tanaka and Tanaka (2011) includes a discussion of how plants use the chlorophyll cycle to regulate the synthesis and destruction of a specific subset of light-harvesting complexes.

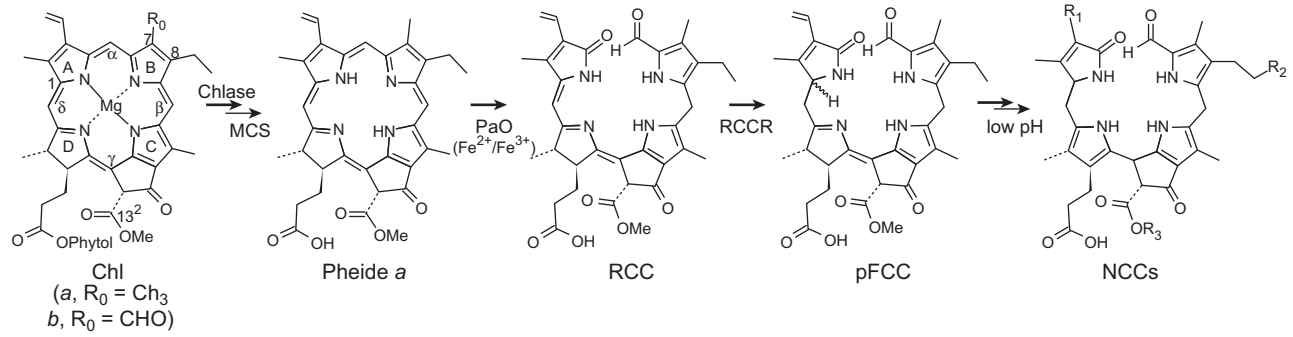
C. Regulation of Chlorophyll Biosynthesis

Of the three steps involved in ALA synthesis, glutamyl-tRNA reduction appears to be the limiting one in chlorophyll biosynthesis (Tanaka and Tanaka, 2007). Current evidence suggests that glutamyl tRNA reductase (GluTR) activity is controlled through feedback regulation by the end products (Meskauskiene *et al.*, 2001). A second negative regulator of tetrapyrrole biosynthesis was later discovered, the FLU protein, which appeared to control chlorophyll biosynthesis by directly interacting with GluTR (Goslings *et al.*, 2004). Other possible regulators include CAO's conversion of chlorophyll *a* to chlorophyll *b* (Tanaka *et al.*, 2001; Tanaka and Tanaka, 2005; Pattanayak *et al.*, 2005). For a more detailed discussion of these regulators the paper by Tanaka and Tanaka (2007) is recommended.

D. Mechanism of Chlorophyll Degradation

The third and final phase of chlorophyll metabolism involves the degradation of chlorophyll (Takamiya *et al.*, 2000; Eckhardt and Grimm, 2004). This phase is central to leaf senescence and fruit ripening, with the reaction mechanism having been well established since the last edition of this book (Matile *et al.*, 1999; Hortensteiner, 1999; Krautler, 2003; Eckhardt *et al.*, 2004). The mechanism of chlorophyll degradation involves its conversion to colorless non-fluorescent catabolites (NCCs) as shown Scheme 2.6.

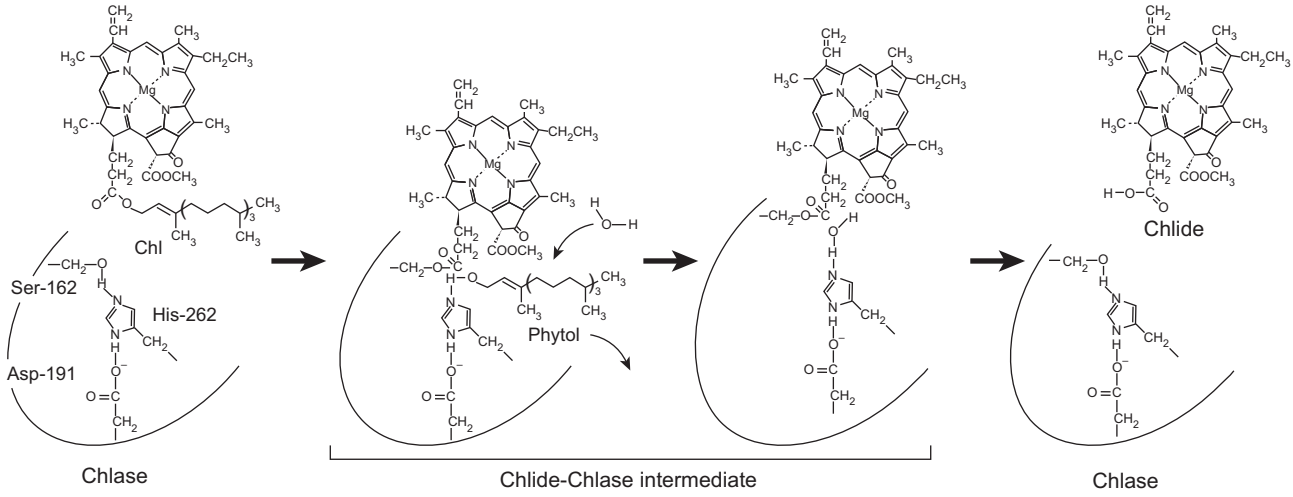
The breakdown of chlorophyll *a* in plants involves four consecutive steps catalyzed by chlorophyllase, Mg-dechelatase, pheophorbide *a* oxygenase and red chlorophyll catabolite reductase, respectively (Harpaz-Saad *et al.*, 2007). The first step is the removal of phytol by the enzyme chlorophyllase (chlorophyll chlorophyllidihydrolase, EC 3.1.1.14), an intrinsic membrane glycoprotein located in the lipid envelope of the thylakoid membranes (Bacon and Holden, 1970; Schoch and Vielwerth, 1983). This enzyme was first discovered a century ago by Willstater and Stoll (1913). Since then, there have been many studies trying to elucidate the mechanisms of reaction. The inhibition of *Euglena gracilis* chlorophyllase by *p*-chloromercuribenzoic acid (PCMB) suggested the possible involvement of cysteine. However, PCMB proved ineffective against *Phaeodactylum tricornium* chlorophyllase (Terpstra, 1977). Later work by Khalyfa *et al.* (1995) found that diisopropyl fluorophosphate, a serine hydrolase inhibitor, inhibited *Paseodactylim* chlorophyllase. These inhibition results suggested that the amino acid residues involved in the chlorophyllase reaction varied with the source of the enzyme. Cloning the chlorophyllase gene from the higher plant *Chenopodium album*, Tsuchiya *et al.* (2003) found that it contained a lipase motif with an active serine residue. Identification of serine, histidine and aspartic acid at the active site of chlorophyllase indicated that it was similar to serine hydrolase.



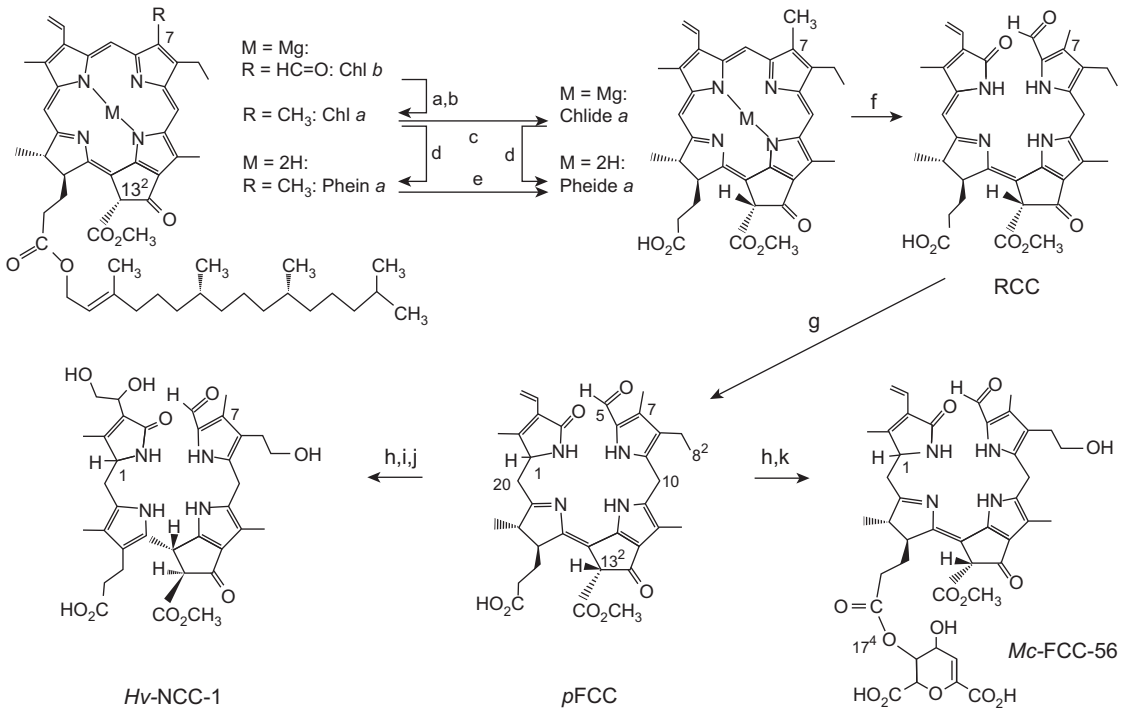
SCHEME 2.6 The pathway of chlorophyll breakdown in higher plants. The chemical constitutions of chlorophyll and chlorophyllide catabolites are shown. Pyrrole rinds (A−D), methine bridges (α−δ), and relevant atoms are labeled. Sites of peripheral modifications as present in different NCCs are indicated (R_1−R_3). Chl = chlorophyll; Pheide *a* = pheophorbide *a*; RCC = red chlorophyll catabolite; pFCC = primary fluorescent chlorophyll catabolite; NCC = non-fluorescent chlorophyll catabolite (Pruzinska *et al.*, 2005).

Based on these results the following mechanism for chlorophyllase was proposed (Scheme 2.7). Azoulay-Shemer *et al.* (2011) found that dual N- and C-terminal processing was involved in the maturation of citrus chlorophyllase.

The enzyme involved in removal of Mg from chlorophyllide *a* with the formation of pheophorbide *a* has been referred to as Mg-dechelatase (Owens and Falkowski, 1988; Ziegler *et al.*, 1988; Shimokawa *et al.*, 1990; Shioi *et al.*, 1991). Using chlorophyllin, Mg-dechelatase was assayed in rape cotyledons by Vicentini *et al.* (1995). The enzyme appeared to be located in a latent form in the thylakoids but present at high levels in presenescent chloroplasts. While there have been a number of studies on Mg-dechelation, none of them has yet identified Mg-dechelatase (Shioi *et al.*, 1996; Tan *et al.*, 2000; Costa *et al.*, 2002; Suzuki and Shioi, 2002). Using chlorophyllide *a*, Shioi *et al.* (1996) found that the Mg-dechelation reaction required a small heat-stable substance, referred to as a metal-chelating substance (MCS). Suzuki and Shioi (2002) used an artificial substrate, chlorophyllin *a*, and showed that a purified Mg-releasing protein from *Chenopodium album* had weak peroxidase activity. This was consistent with an earlier study by Azuma *et al.* (1999), who showed that horseradish peroxidase exhibited Mg-dechelating activity. Subsequent research using extracts of *C. album* by Kunieda *et al.* (2005), however, found that peroxidase and glutathione *S*-transferase could only release magnesium from the artificial substrate chlorophyllin *a*. This eliminated their possible role in the degradation of chlorophyll, as only the purified low-molecular-mass protein exhibited Mg-dechelating activity with the native substrate chlorophyllide *a*.



SCHEME 2.7 A possible model for the catalytic mechanism of the recombinant *Chenopodium album* chlorophyllase (CaCLH) (Tsuchiya *et al.*, 2003).



SCHEME 2.8 Representative structural outline of major catabolites delineating the major paths of chlorophyll breakdown in higher plants (Krautler and Hortensteiner, 2006; Moser *et al.*, 2009). (Hortensteiner and Krautler, 2011).

Pheophorbide *a* oxygenase (PAO), identified in the soluble protein fraction of *C. alba* by Shioi *et al.* (1995), catalyzes the conversion of pheophorbide *a* to pyropheophorbide *a*. PAO, a Rieske-type iron−sulfur oxygenase (Pruzinska *et al.*, 2003, 2005), is the key enzyme responsible for opening the chlorine macrocycle of pheophorbide *a* (Hortensteiner and Krautler, 2011). Unsuccessful attempts to biochemically characterize PAO in isolated chloroplast membranes led to the identification of a second enzyme, red chlorophyll catabolite reductase (RCCR). Only after the addition of stromal proteins, which contained RCCR, could PAO activity be determined. Together these two enzymes catalyzed the formation of a non-polar fluorescent chlorophyll catabolite (FCC) as primary FCC (pFCC). The required opening of the macrocycle of pheophorbide *a* by the PAO oxygenase formed the elusive red chlorophyll catabolite (RCC), which was then reduced by RCCR to FCC. pFCC was then further modified by unidentified hydroxylating enzymes to a variety of colorless end products (Scheme 2.8).

1. Chlorophyll Degradation: Processing and Storage

The retention of chlorophyll is generally used as a measure of quality in green vegetables as chlorophyll degradation occurs in damaged tissue during blanching and processing (Sweeney and Martin, 1961; Heaton and Marangoni, 1996; Tijkens *et al.*, 2001). Several pathways have been proposed for the loss of chlorophyll during processing and storage of fruits and vegetables. In addition to enzymes, weak acids, oxygen, light, and heat can all result in the breakdown of chlorophyll. This loss of green color can be undesirable and such changes need to be minimized. One of the main reactions is replacement of the Mg^{2+} atom in chlorophyll by hydrogen under acidic conditions with the formation of pheophytin (Minguez-Mosquera *et al.*, 1989). The latter pigment is associated with a color change from a bright green to a dull olive green. This reaction was first recognized by Campbell (1937) to cause discoloration in stored frozen peas. The influence of pH on the conversion of chlorophyll to pheophytin is generally considered to be the main cause of discoloration of foods during processing. Thus, vegetables turn a dull olive green when heated or placed in an acidic environment (Gold and Weckel, 1959; Gunawan and Barringer, 2000). As a consequence, efforts to maintain chlorophylls during heat processing included pH control, high-temperature short time processing, or a combination (Gupte and Francis, 1964; Buckle and Edwards, 1970; Schwartz and Lorenzo, 1991).

The rate of conversion to pheophytin was shown to be first order with respect to the acid concentration (Joslyn and Mackinney, 1938). The formation of pheophytin has been the subject of a large number of studies (Gupte *et al.*, 1964; Hermann, 1970; LaJollo *et al.*, 1971; Robertson and Swinburne, 1981). A linear relationship was reported by Walker (1964) between the appearance and pheophytin formation for frozen beans stored for up to 1 year. LaJollo *et al.* (1971) noted that the formation of pheophytin was the predominant reaction at water activity (a_w) levels greater than 0.32 in freeze-dried, blanched spinach purée stored at 37°C and 55°C under nitrogen and air. Chlorophyll *a* was degraded far more rapidly than chlorophyll *b*, by a factor of 2.5−3.0, consistent with earlier reports (Schanderl *et al.*, 1962; Gupte *et al.*, 1964). LaJollo *et al.* (1971) reported a linear relationship between a_w and log time for a 20% loss of chlorophyll (Figure 2.8). These early studies primarily used spectrophotometric and colorimetric methods to establish the kinetics of chlorophyll degradation. Later, researchers used HPLC to measure chlorophylls *a* and *b* (Steet and Tong, 1996; Mangos and Berger, 1997; Weemaes *et al.*, 1999). Using HPLC, Koca *et al.* (2006) confirmed the first order degradation of chlorophyll *a* and *b* in blanched green peas over a temperature range of 70−100°C in buffered solutions at pH 5.5, 6.5, and 7.5. Using the CIE-$L^*a^*b^*$ color measuring system, they found a significant correlation between the change in visual color parameters ($-a$, $-b$, and h values) and chlorophyll degradation.

Besides pheophytinization, chlorophyllase converts chlorophylls to chlorophyllides with the loss of the phytol group. The combined action of chlorophyllase and acid results in the loss of Mg^{2+} and phytol group with the formation of pheophorbides (White *et al.*, 1963). Pheophorbide was also found to be the major degradation product during the brining of cucumbers (Jones *et al.*, 1961, 1963). Several new products were identified by Schwartz *et al.* (1981) in heated spinach purée, including pyropheophytins *a* and *b*. These were formed from the corresponding pheophytins as a result of the loss of the carbomethoxy group and separated by HPLC. Schwartz and von Elbe (1983) examined the formation of pyropheophytins in heated spinach. Pheophytins *a* and *b* were both detected after heating at 121°C for up to 15 minutes, but then a decline in these pigments occurred. Further heating resulted in the formation of pyropheophytin with *a* detected after 4 minutes while *b* was not observed until after 15 minutes. A sample of blanched spinach purée heated for almost 2 hours at 126°C produced only pyropheophytins *a* and *b*. No further changes were noted, indicating that these were the final products of degradation. The olive-green color associated with canned vegetables was attributed to pyropheophytins. These pigment derivatives probably accounted for the unidentified pigments reported by Buckle and Edwards (1969) and LaJollo *et al.* (1971) to represent 20−30% of the total pigments. The inability of these researchers to detect pyropheophytins was due to the absence of high-resolution chromatographic techniques required to separate these derivatives. Schwartz and von Elbe (1983) proposed the following kinetic sequence to explain the formation of pyropheophytins:

$$\text{Chlorophyll} \rightarrow \text{Pheophytin} \rightarrow \text{Pyropheophytin}$$

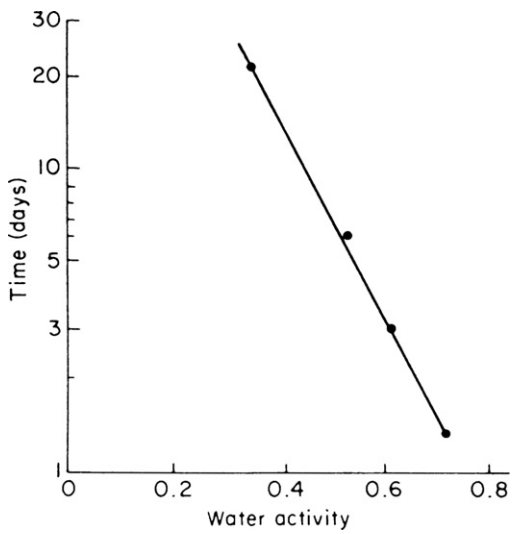
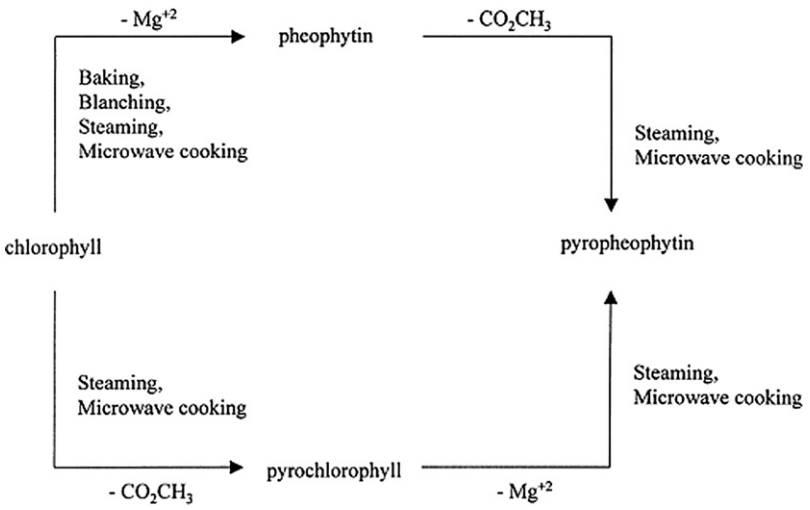


FIGURE 2.8 Time required for 20% loss of chlorophyll in spinach at different water activities (37°C in air) (La Jollo *et al.*, 1971). *(Copyright © by Institute of Food Technologists.)*

A later study by Teng and Chen (1999) examined the changes in chlorophylls and their derivatives during the heating of spinach leaves using HPLC with a photodiode array or positive ion fast atom bombardment mass spectrometry (FAB-MS). They showed the first order degradation of both chlorophyll *a* and *b* and that the rate constant was greater for microwave cooking and blanching than for steaming and baking. Chlorophyll epimers and pheophytins were the major degradation products formed during baking and blanching while pyrochlorophylls *a* and *b* were detected after steaming for 30 minutes or microwave cooking for 1 minute. Microwave cooking appeared to favor the formation of pyrochlorophylls *a* and *b*, while steaming favored the formation of pyropheophytins *a* and *b*. The formation of these chlorophyll derivatives during the heating of spinach leaves was described by these researchers in Scheme 2.9.

To preserve the desirable fresh green color of vegetables, the potential use of metallochlorophyll complexes has been examined. A regreening phenomenon was observed involving the formation of such complexes resulting from the introduction of copper (Cu) and zinc (Zn) in the chlorophyll pyrrole ring. These appear to form a strong bond that is more resistant to acid and heat than the normal magnesium (Mg^{2+})-containing chlorophyll (Humphrey, 1980). Pheophytin *a*, pyropheophytin *a*, and phephorbide *a* were found to be much more reactive with zinc than were the corresponding *b* forms (von Elbe *et al.*, 1986; LaBorde and von Elbe, 1990). Several patents were issued for improving the color of green vegetables by blanching or packing green vegetables in zinc or copper solutions (Leake and Kirk, 1992; LaBorde and von Elbe, 1996). A study by Canjura *et al.* (1999) found an improvement in the green color of aseptically processed fresh and frozen green peas when blanched in a solution of $ZnCl_2$ that may prove to be viable for preserving the green color of vegetables.

Holden (1965) also attributed decoloration of chlorophyll in legume seeds to the oxidation of fatty acids by lipoxygenase (LOX). This reaction was coupled with the degradation of fatty acid hydroperoxides and required a thermolabile factor to be present in the crude extract. Subsequent research by Zimmerman and Vick (1970) identified an enzyme, linoleate hydroperoxide isomerase, as the heat-labile factor. The bleaching effect on chlorophyll was attributed to an oxidation−reduction reaction in which the ketohydroxy fatty acid, the isomerized product formed by hydroperoxide isomerase, and a portion of the conjugated double-bond system of chlorophyll were involved. Imamura and Shimizu (1974) disproved the involvement of hydroperoxide isomerase and confirmed the role of LOX in chlorophyll bleaching. Later researchers confirmed that different LOX isoenzymes played a role in bleaching chlorophyll and carotenes (Grosch *et al.*, 1976; Ramadoss *et al.*, 1978). The participation of the different LOX isoenzymes in carotene and chlorophyll bleaching has been the subject of a number of investigations (Hilderbrand and Hymovitz, 1982; Reynolds and Klein, 1982; Cohen *et al.*, 1984; King and Klein, 1987). The effect of soybean LOX-1 on wheat chloroplasts investigated by Kockritz *et al.* (1985) suggested that this enzyme, which selectively attacks free fatty acids, may be involved in senescence and chloroplast breakdown. LOX activity has been reported previously in chloroplasts of peas (Borisova and Budnitskaya, 1975; Douillard and Bergeron, 1978). Because of its role in the deterioration of vegetable quality, LOX was considered to be a possible indicator enzyme for optimizing blanching prior to freezing. However, Gokmen *et al.* (2005) clearly showed that peroxidase (POD) was more heat stable than LOX, so the inactivation of POD was a better indicator for the adequacy of blanching.

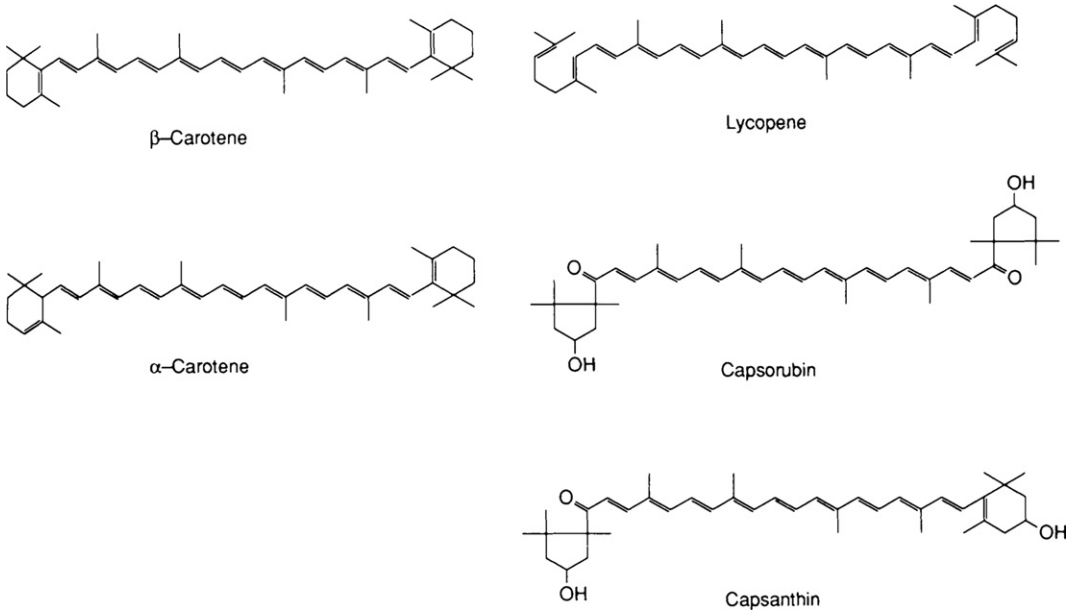


SCHEME 2.9 Formation pathway of pyrochlorophylls and their derivatives in spinach leaves during heating (Teng and Chan, 1999).

E. Carotenoids

During the maturation of many fruits there is a change in color from green to orange or red. This is due to the loss in chlorophyll and the unmasking and synthesis of carotenoids (MacKinney, 1961). Such pigmentation changes are accompanied by structural changes in the chloroplasts. The granal−integranal network, in particular, becomes disorganized, resulting in the formation of chromoplasts (Thomson, 1966; Spurr and Harris, 1968; Camara and Brangeon, 1981). The chromoplasts no longer contain chlorophyll or photosynthetic pigments but become the major site for carotenoid biosynthesis (Camara and Brangeon, 1981).

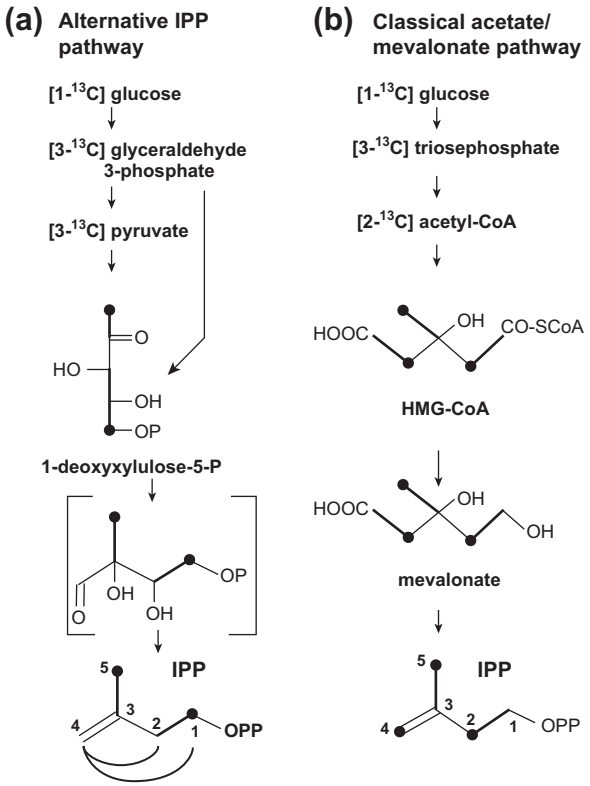
Carotenoids are C_{40} isoprenoid compounds composed of isoprene units joined head to tail to form a system of conjugated double bonds (Eskin, 1979). They are classified into two groups, carotenes and xanthophylls. The carotenes are structurally related to hydrocarbons while the xanthophylls include the corresponding oxidized derivatives (hydroxy, epoxy, and oxy compounds) and are frequently esterified. Examples of carotenes are α- and β-carotenes in carrots and lycopene in tomatoes, while xanthophylls include capsanthin and capsorubin found in red pepper.



Carotenoids are synthesized within the chromoplast from isopentyl pyrophosphate (IPP). At one time IPP biosynthesis was thought to occur via a single pathway involving acetate/mevalonate (Britton, 1982). However, a second pathway was discovered in aubacteria, green algae, and higher plants in which IPP was formed from glyceraldehyde 3-phosphate and pyruvate (Lichtenthaler *et al.*, 1997a, b). Both pathways are present in higher plants but differ in their localization. The mevalonate pathway enzymes are found in the cytosol, while the glyceraldehyde 3-phosphate pathway, referred to as the 1-deoxy-D-xylulose-5-phosphate pathway (DXP), operates in the plastid (Scheme 2.10).

It remained somewhat unclear whether acetyl-CoA was synthesized within the chloroplast or was of extraplastidic origin. Grumbach and Forn (1980) showed quite clearly that acetyl-CoA was formed within the chloroplast and could synthesize carotenoids autonomously. Chromoplasts from both red peppers and red daffodils exhibited an ability to synthesize carotenoids from isopentyl phosphates (Beyer *et al.*, 1980; Camara *et al.*, 1982). The presence of enzymes capable of synthesizing acetyl-CoA suggested that a similar autonomy existed in the chromoplasts capable of synthesizing carotenoids.

The formation of geranylgeranyl pyrophosphate from mevalonic acid (MVA) involves phosphorylation by mevalonate kinase (ATP:mevalonate 5-phosphotransferase, EC 27.1.3.6). This enzyme has been identified in many plants, including pumpkin seedlings (Loomis and Battaille, 1963), green leaves and etiolated cotyledons of French beans (*Phaseolus vulgaris*) (Rogers *et al.*, 1966; Gray and Keckwick, 1969, 1973), orange juice vesicles (Potty and Breumer, 1970), and melon cotyledons (*Cucumis mello*) (Gray and Keckwick, 1972). Phosphorylation of MVA-5P to MVA-5 pyrophosphate (MVA-5PP) is then catalyzed by 5-phosphomevalonate kinase (ATP:phosphomevalonate phosphotransferase, EC 2.7.4.2). MVA-5 PP is then decarboxylated by pyrophosphomevalonate decarboxylase [ATP:5-pyrophosphomevalonate carboxylyase (dehydrating), EC 4.1.1.33]. This enzyme catalyzes a bimolecular

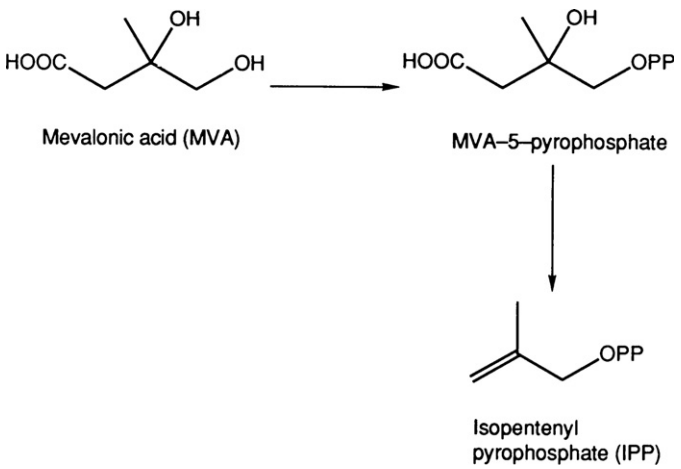


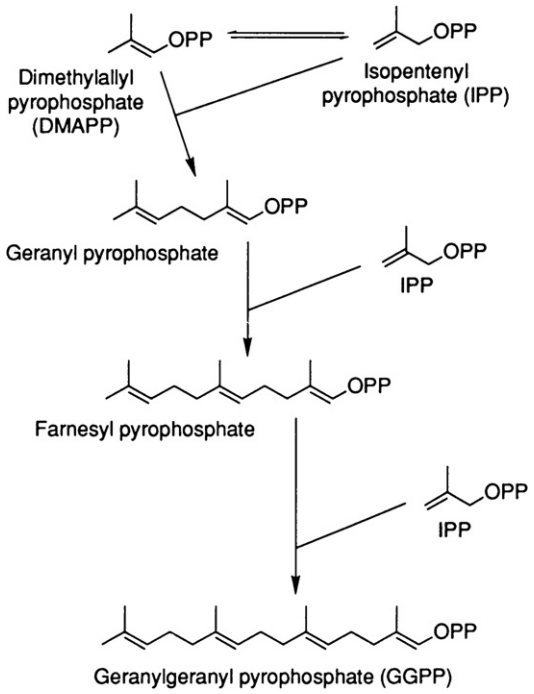
SCHEME 2.10 Labeling of isopentyl diphosphate (IPP) from [^{13}C]glucose via (a) a novel alternative and (b) the classical acetate/mevalonate pathway of IDPP biosynthesis (Lichtenthaler *et al.*, 1997b).

reaction in which ATP and 5-pyrophosphomevalonate are converted to isopentyl pyrophosphate, ADP, phosphate, and carbon dioxide (Scheme 2.11).

Isomerization of isopentyl pyrophosphate to dimethylallyl pyrophosphate is catalyzed by isopentyl pyrophosphate isomerase (EC 5.3.3.2). The double bond is isomerized from position 3 in isopentyl pyrophosphate to position 2 in dimethylallyl pyrophosphate. This enzyme was isolated from pumpkin fruit by Ogura *et al.* (1968). One molecule of dimethylallyl pyrophosphate then condenses with one, two, or three molecules of isopentyl pyrophosphate, leading to the formation of geranylgeranyl pyrophosphate (Scheme 2.12). The last reactions are catalyzed by a group of enzymes referred to as prenyl transferases. The enzyme responsible for the synthesis of farnesyl pyrophosphate was partially purified from pumpkin seed (Eberhardt and Rilling, 1975).

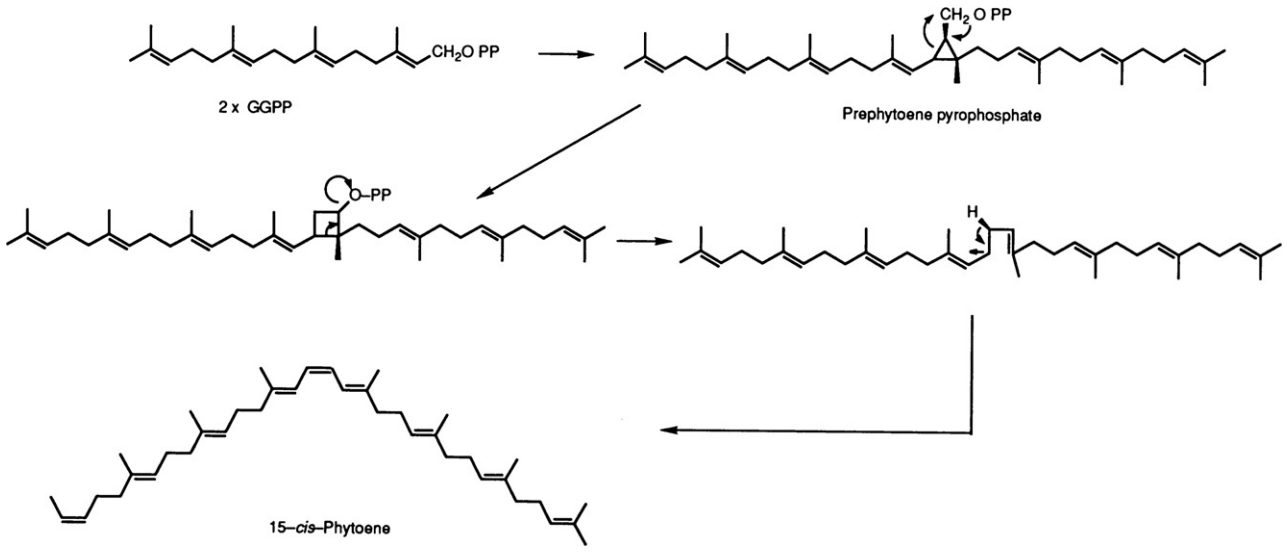
SCHEME 2.11 Formation of IPP from mevalonic acid.



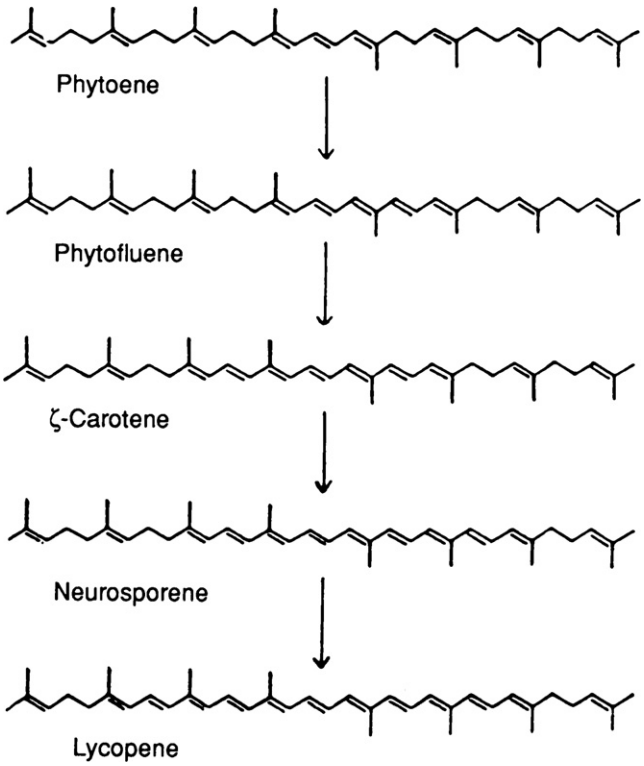


SCHEME 2.12 Geranylgeranyl pyrophosphate biosynthesis from IPP.

The formation of the first C_{40} hydrocarbon 15-*cis*-phytoene results from condensation of two molecules of geranylgeranyl pyrophosphate (C_{20}) (Scheme 2.13). The intermediate in this reaction, prephytoene pyrophosphate, loses a proton, which results in a double bond at the C15 position. Maudinas *et al.* (1975) identified a soluble enzyme system from tomato fruit plastids which was capable of synthesizing *cis*-phytoene from isopentyl pyrophosphate. Phytoene is quite colorless and is converted to colored carotenoids by a series of desaturation steps which produce a conjugated double-bond system. Unlike phytoene, which is a 15-*cis* isomer, the colored carotenoids are all *trans* so that isomerization to the *trans* form must take place during the desaturation process. The mechanism involves the loss of hydrogen by trans-elimination and may be mediated by an enzyme complex in the membrane, possibly involving metal ions or cytochromes in a simple electron transfer system (Britton, 1979). The sequential desaturation of



SCHEME 2.13 Mechanism of phytoene biosynthesis (Britton, 1982).



SCHEME 2.14 Sequence of desaturation reactions leading from phytoene to lycopene (Britton, 1982).

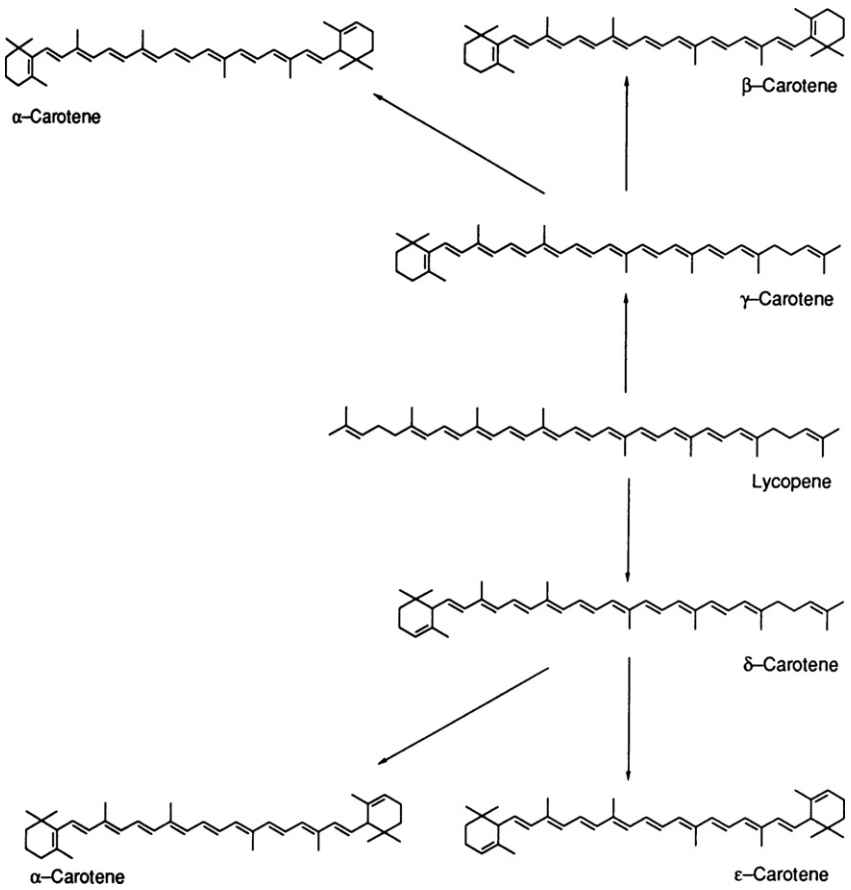
phytoene to lycopene was proposed by Porter and Lincoln (1950) and is shown in Scheme 2.14. A similar enzyme system was reported by Qureshi *et al.* (1974) in tangerine mutant tomatoes capable of converting *cis*-β-carotene into all-*trans* carotene. The conversion of neurosporene into lycopene has been found in fungal systems only (Davies, 1973; Bramley *et al.*, 1977).

The final step in carotenoid biosynthesis is cyclization with the formation of at least one or two cyclic end groups in the carotenoids (Scheme 2.15). The conversion of lycopene to α-, β, and γ-carotenes was demonstrated in the presence of soluble enzymes from tomato fruit plastids and spinach chloroplasts by Kuwasha *et al.* (1969). Cyclization was shown to be inhibited by nicotine and CPTA [2-(4-chlorophenyltrio)triethyl ammonium chloride] with the accumulation of lycopene in citrus fruits treated with these inhibitors (Britton, 1982). Following cyclization, oxygen is incorporated as a hydroxyl group at C3 or an epoxide at the 5,6 position (Takeguchi and Yamamoto, 1968; Britton, 1976). The latter involves a series of reactions referred to as the xanthophyll cycle.

Camara *et al.* (1982) examined the site of carotenoid biosynthesis in chromoplasts of semi-ripened pepper (*Capsicum annium* L.) fruits. Incubation of [1-^{14}C] isopentyl diphosphate with different chromoplast fractions showed that the membrane was incapable of synthesizing carotenoids, while the stroma synthesized the first colorless carotenoid, phytoene (Table 2.4). Thus, phytoene synthetase, the enzyme responsible, could be a useful indicator of the chromoplast stroma. Increased incorporation of the labeled substrate in the presence of chromoplast membranes was attributed to their desaturation and cyclization systems, which produced colored carotenoids. This study showed that the enzymes involved in carotenoid biosynthesis were compartmentalized in the chromoplasts. The stroma synthesized phytoene synthesis, which underwent desaturation and cyclization to the colored carotenoids in the membrane. These researchers proposed that a protein carrier transferred phytoene to the chromoplast membrane, or alternatively that phytoene synthetase itself was bound to the membrane, where it discharged phytoene for further reactions.

1. Carotenoid Changes during Ripening

Ebert and Gross (1985) examined the carotenoid pigments in the peel of ripening persimmon (*Diospyros kaki* cv. 'Triumph'). A steady decline in chlorophyll (*a* and *b*) was observed during the course of ripening which disappeared



SCHEME 2.15 Overall scheme for the biosynthesis of bicyclic carotenes from neurosporene (Britton, 1982).

in the harvest-ripe fruit (Table 2.5). The chloroplast carotenoids (α- and β-carotenes, lutein, violaxanthin, and neoxanthin) decreased, followed by the gradual synthesis of the chromoplast carotenoids (cryptoxanthin, antheraxanthin, and zeaxanthin). Ikemefuna and Adamson (1985) monitored changes in the chlorophyll and carotenoid pigments of ripening palm fruit (*Elaeis quineeris* 'Palmal') and noted a similar degeneration of chloroplasts and formation of chromoplasts. These changes were accompanied by increase in carotenogenesis with the formation of α- and β-carotenes as the major pigments in the ripe fruit.

Farin *et al.* (1983) examined the change in carotenoids during the ripening of the Israeli mandarin hybrid 'Michal' (*Citrus reticulata*). This particular fruit is the most highly colored of the citrus fruits with a bright-reddish color. Total chlorophyll decreased rapidly in the peel and completely disappeared at the ripening stage (Table 2.6). The total carotenoids decreased at the color break stage due to a decline of the chloroplast carotenoids, δ-carotene, lutein, violaxanthin, and neoxanthin. A marked rise in carotenogenesis followed, with an increase in the chromoplast carotenoids, cryptoxanthin and C_{30} apocarotenoids. The two C_{30} apocarotenoids, β-citraurin and β-citraurinene, accounted for 26.1% and 9.9%, respectively, of the total carotenoids in the ripe fruit. Their formation appeared to require asymmetric degradation of a C_{40} fragment from the side of the C_1 carotenoid, cryptoxanthin.

Gross *et al.* (1983) studied changes in pigments and ultrastructure during the ripening of pummelo (*Citrus grandis* Osbech). Chlorophyll decreased from 90 μg/g at the unripe (green) stage to 11 μg/g in the ripe pale-yellow fruit, totally disappearing by the fully ripe stage. The chloroplast carotenoids, β-carotene, lutein, violaxanthin, and neoxanthin, all decreased during ripening, with the β-carotene totally disappearing. Accumulation of phytofluene was evident at the color break, reaching 67% of the total carotenoids in the ripe fruit. Besides phytofluene, other chromoplast carotenoids were detected, including δ-carotene, neurosporene cryptoxanthin, and cryptoflavin.

These studies showed that compositional changes in carotenoids during ripening reflect the transformation of chloroplasts to chromoplasts. Earlier work by Eilati *et al.* (1975) on ripening Shamouti 'orange peel' showed that parallel transformations occurred irrespective of whether the fruit was attached to or detached from the tree. The transformation of chloroplasts to chromoplasts during degreening of citrus fruits was reported by Huff (1984) to be regulated by the

TABLE 2.5 Carotenoid Changes in the Peel of Persimmon (*Dlospyros kaki cv.* 'Triumph') during Postharvest Ripening

	Ripening Stage		
	Harvest-ripe	**Intermediate**	**Fully Ripe**
Total carotenoids (μg/g fr. wt)	128.0	366.0	491.0
Carotenoid pattern (% of total carotenoids)			
Phytofluene	–	–	0.4
α-Carotene	1.6	1.2	1.0
β-Carotene	9.4	7.6	6.7
Mutatochrome	–	0.7	–
γ-Carotene	–	0.4	–
Lycopene	1.1	0.5	8.2
β-Cryptoxanthin	29.2	50.0	48.2
Cryptoxanthin 5,6-epoxide	0.9	1.2	1.9
Cryptoflavin	0.7	2.1	2.9
Lutein	12.4	5.5	4.1
Zeaxanthin	9.3	9.7	5.9
Mutatoxanthin	0.8	4.7	1.8
Isolutein	0.5	–	0.3
trans-Antheraxanthin	5.4	2.0	4.8
cis-Antheraxanthin	6.2	2.2	2.3
Luteoxanthin	1.7	1.8	1.9
trans-Violaxanthin	6.9	3.7	3.8
cis-Violaxanthin	6.7	1.5	2.0
Neoxanthin	7.2	5.2	3.8

From Ebert and Gross (1985). Reprinted with permission. Copyright © by Pergamon Press.

accumulation of sugar in the epicarp. The reverse transformation associated with the regreening of certain citrus species was found to be accompanied by the disappearance of sugars. This regreening phenomenon of *Citrus sinensis* fruit epicarp observed by Thomson *et al.* (1967) was attributed to the reversion of chromoplasts to chloroplasts. Thus, sucrose promoted the formation of chromoplasts while nitrogen stabilized the chloroplasts by retarding the degreening process.

2. Carotenoid Degradation: Processing and Storage

Carotenoids impart most of the yellow and orange colors to fruits and vegetables such as pineapples and carrots. The unsaturated nature of carotenoids renders them particularly susceptible to isomerization and oxidation, resulting in a loss of color which is most pronounced following oxidation. The latter can be brought about by the action of lipoxygenase, which can bleach carotenoids (Eskin *et al.*, 1977). From recent research, it appears that differences in the abilities of lipoxygenases from fruits and vegetables to oxidize carotenoids are due to the isoenzymes present. Lipoxygenase isoenzymes are classified as either type 1 or type 2 depending on their pH optimum and product specificity. Of these isoenzymes, lipoxygenase-2 exhibits a more acidic pH optimum and is involved in cooxidation reactions leading to pigment discoloration (Klein and Grossman, 1985). For example, pea lipoxygenase-2 was shown by Arens *et al.* (1973) to be an effective oxidizer of carotenoids. Chepurenko *et al.* (1978) attributed carotenoid bleaching to the combined effect of the lipoxygenase isoenzymes in peas and not just to lipoxygenase-2. Yoon and

TABLE 2.6 Pigment Distribution in the Flavedo of a Mandarin Hybrid (*Citrus reticulate cv.* 'Michal') during Ripening

	Peel		
	Green	**Color Break**	**Ripe**
Fruit diameter (cm)	4.70	4.85	5.10
Chlorophyll *a* (μg/g)	240.0	52.6	–
Chlorophyll *b* (fr. wt)	86.0	15.8	–
Total carotenoids	143.4	51.0	174.1
Carotenoid pattern (% of total carotenoids)			
Phytofluene	–	5.2	3.1
α-Carotene	9.7	2.4	0.2
β-Carotene	6.9	2.5	0.3
ζ-Carotene	–	–	0.4
δ-Carotene	–	–	0.1
Mutatochrome	–	–	0.5
Lycopene	–	–	–
β-Apo-8′-carotenal	0.7	0.7	1.3
α-Cryptoxanthin	1.9	–	–
β-Cryptoxanthin	–	3.1	6.4
Cryptoxanthin 5,6-epoxide	–	–	0.4
Cryptoxanthin 5′,6′-epoxide	–	–	0.3
β-Citraurinene	–	9.5	9.9
β-Citraurin	–	12.3	26.1
Lutein	23.5	12.8	2.6
Zeaxanthin	3.9	1.6	1.0
Mutatoxanthin	1.5	0.2	0.2
trans-Antheraxanthin	3.6	4.3	1.8
cis-Antheraxanthin	–	–	2.5
Luteoxanthin	5.8	5.6	9.1
trans-Violaxanthin	14.0	11.7	9.8
cis-Violaxanthin	11.0	18.2	19.8
trans-Neoxanthin	11.7	6.6	4.2
cis-Neoxanthin	3.6	3.3	–
Neochrome	–	–	–
trans-Trollixanthin	–	–	–
Trollichrome	–	–	–
Unknown	2.2	–	–

Adapted from Farin *et al.* (1983). Reprinted with permission.

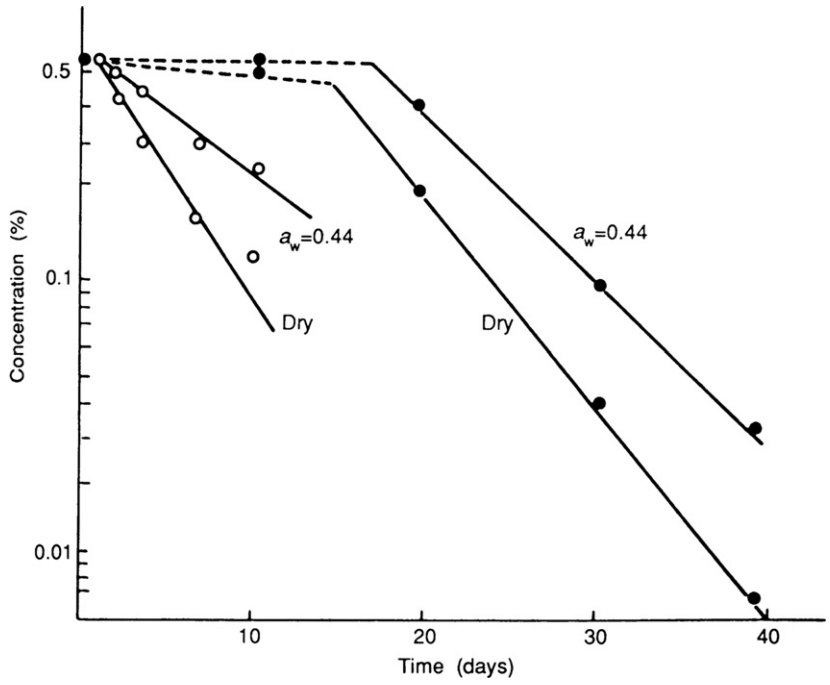


FIGURE 2.9 Decoloration of β-carotene in model systems. Dry refers to systems held over solid $CaCl_2$; $a_w = 0.44$ to systems held over saturated K_2CO_3 (○: control; ●: BHT-butylated hydroxytoluene) (Chou and Breene, 1972). *(Copyright © by Institute of Food Technologists.)*

Klein (1979) showed definite differences between the rate of carotenoid oxidation for these two pea lipoxygenase isoenzymes. These enzymes were also involved in the biosynthesis of traumatic acid, a wound-healing hormone, as well as the plant growth regulator jasmonic acid (Zimmerman and Coudron, 1979; Vick and Zimmerman, 1983).

Carotenoids are extremely susceptible to non-enzymatic oxidation in dehydrated fruits and vegetables. For example, powdered, dehydrated carrots were reported by MacKinney *et al.* (1958) to lose 21% of their carotenoids when stored in air. Of particular importance to their stability is the amount of moisture present in dehydrated products. The effect of a_w on the degradation of β-carotene in model systems was studied by Chou and Breene (1972). These researchers showed that at a_w of 0.44 the oxidative decoloration of β-carotene was reduced compared to the corresponding dry system with or without the presence of the antioxidant butylated hydroxytoluene (BHT) (Figure 2.9). It is clear from this study that water acts as a barrier to oxygen diffusion.

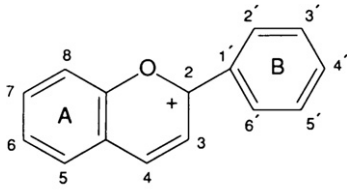
When carotenoids are heated in the absence of air some of the *trans* double bonds undergo isomerization to the corresponding *cis* isomers. Acids also catalyze isomerization from the all-*trans* form to the corresponding *cis* isomer. The change in shape associated with the *cis* isomers reduces the resonance in the molecule as well as the color intensity. These reactions were responsible for the difference in quality between canned and fresh pineapple (Singleton *et al.*, 1961). A change in the spectrum of the extracted carotenoid pigments was reported for canned pineapples, including loss of a peak at 466 nm together with peaks of shorter wavelengths. This spectral shift, although too subtle for the human eye, caused a slight color shift from orange−yellow in the fresh pineapple to a more lemon−yellow in the canned product. This shift was attributed to isomerization of carotenoids with 5,6-epoxide groups to the corresponding 5,6-furanoid oxides. In the intact fruit the natural cell vacuole acids have a catalytic effect by coming into contact with the carotenoid-bearing plastids when cell membranes are disrupted during handling or processing. Several studies examined the effect of cooking carrots on the formation of *cis* isomers. Klaui (1973), however, suggested that the effects of cooking on color and carotene content were insignificant.

F. Anthocyanins

Anthocyanins are responsible for the attractive pink, red, purple, and blue colors of flowers, leaves, fruits, and vegetables (Harborne, 1967). These are water-soluble pigments that accumulate in the epidermal cells of fruits as well as in roots and leaves (Timberlake and Bridle, 1975, 1982; Harborne, 1976). These pigments are formed as the fruit matures and ripens, as is evident for strawberries (Fuleki, 1969), certain varieties of cherries, raspberries, cranberries, and apples (Zapsalis and Francis, 1965; Proctor and Creasy, 1971; Cansfield and Francis, 1970; Craker

and Wetherbee, 1973; Bishop and Klein, 1975), black grapes (Liao and Luh, 1970), and blueberries (Suomalainen and Keranen, 1961), as well as in such vegetables as red cabbage, russet potato, radishes, and red onion (Fuleki, 1971; Small and Pecket, 1982). The primary role of anthocyanins is that of an insect or bird attractant for pollination of flowers and for fruit seed dissemination by animals (Brouillard, 1983).

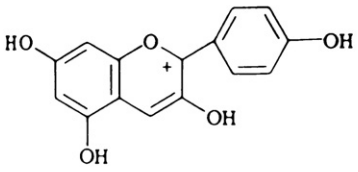
Anthocyanins are flavonoid pigments whose structure is based on the phenylpropanoid nucleus. They occur in nature as glycosides in which the aglycone forms, or anthocyanidins, are substituted flavylium salts. The structural formula of the flavylium cation is as follows:



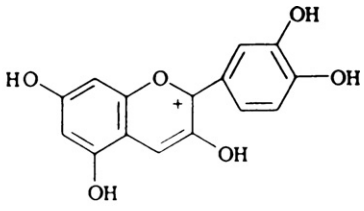
Flavylium cation

The anthocyanidins, or aglycones, are somewhat less stable than the corresponding glycosides. The latter are composed primarily of 3-glycosides and 3,5-glycosides. The major anthocyanidins, or aglycones, in fruits are listed in Scheme 2.16. D-Glucose, D-lactose, l-rhamnose, and D-xylose are the main sugars linked to the anthocyanidin at the 3-position. The intensity of the color is determined by the nature of the pigment, its concentration, and pH, as well as the presence of pigment mixtures, copigments, and certain metallic ions (Brouillard, 1983). Anthocyanins are dissolved in the aqueous vascular sap in the plant cell, which is slightly acidic. These pigments tend to be more stable under these acidic conditions. The different shades of colors of anthocyanidins reflect the nature of their

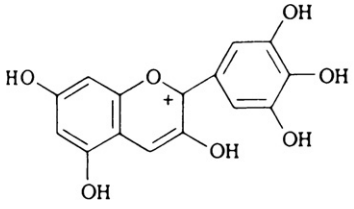
SCHEME 2.16 Major food anthocyanins.



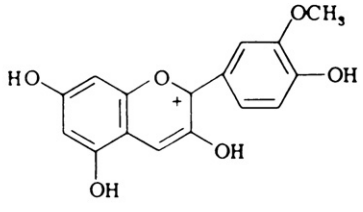
Pelargonidin (3, 5, 7, 4´-tetrahydroxyflavylium cation)



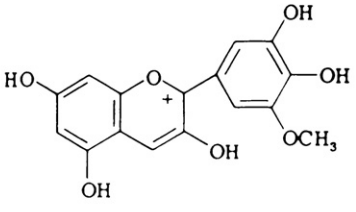
Cyanidin (3, 5, 7, 3´, 4´-pentahydroxyflavylium cation)



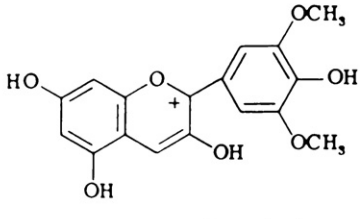
Delphinidin (3, 5, 7, 3´, 4´, 5´-hexahydroxyflavylium cation)



Peonidin (3, 5, 7, 4´-tetrahydroxy-3´-methoxyflavylium cation)



Petunidin (3, 5, 7, 3´, 4´-pentahydroxy-5´-methoxyflavylium cation)



Malvidin (3, 5, 7, 4´-tetrahydroxy-3´, 5´-dimethoxyflavylium cation)

TABLE 2.7 Distribution of Anthocyanins in Some Edible Fruit

Anthocyanidin	Fruit
Cyanidin	Blackberry, rhubarb
Cyanidin, delphinidin	Blackcurrant
Cyanidin, peonidin	Cherry
Cyanidin, pelargonidin	Strawberry

hydroxylation and methoxylation patterns. An increase in hydroxylation is accompanied by an increase in the blue hue, whereas methoxylation enhances the red color (Braverman, 1963). The distribution of anthocyanidins in some edible fruits is shown in Table 2.7.

1. Biosynthesis of Anthocyanins

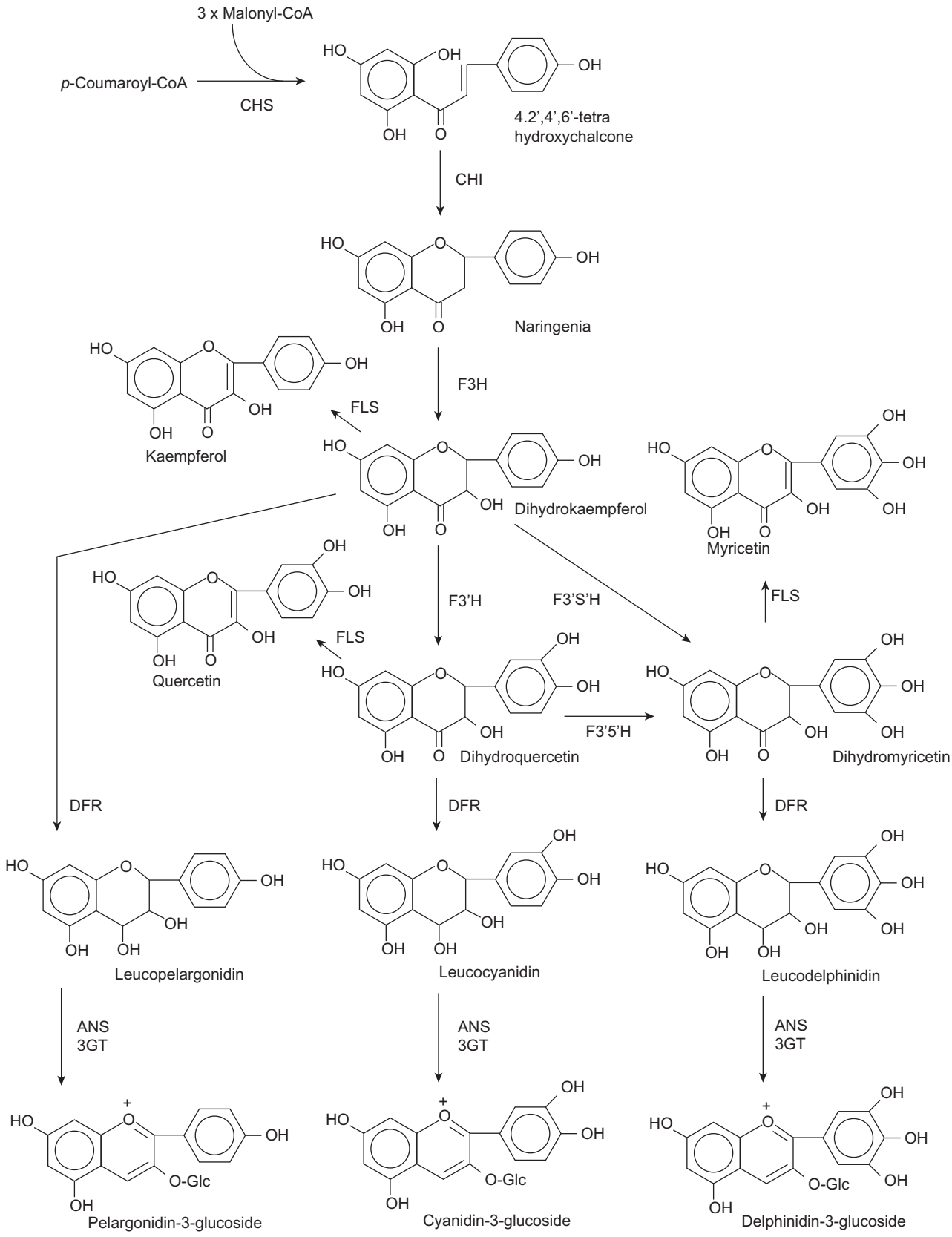
The biosynthesis of anthocyanins is well established (Mol *et al.*, 1989; Forkmann, 1991), with a generalized scheme shown in Scheme 2.17 (Holton and Cornish, 1995). The synthesis of aromatic amino acids is achieved in plants via the shikimic acid pathway. The synthesis of all flavonoids, including anthocyanins, involves three key enzymes, chalcone synthase (CHS), chalcone isomerase (CHI), and flavonoid 3-hydroxylase (F_3H) (Gou *et al.*, 2011). These enzymes appear to be present as multiprotein enzyme complexes (referred to as metabolons) channeling the substrate through specific subpathways (Winkel, 2004; Jorgensen *et al.*, 2005). For example CHS, CHI, F_3H, and dihidroflavonoid-4-reductase (DFR) are all located in the endoplasmic reticulum in *Arabidopsis* (Lepiniec *et al.*, 2006). CHS is responsible for the stepwise condensation of the phenylpropanoid precursor, *p*-coumaryl-CoA, with three molecules of malonyl CoA to form tetrahydroxychalcone. The next enzyme, CHI, isomerizes the yellow-colored chalcone to the colorless naringenin. The third step, the formation of dihydrokaemferol, is catalyzed by F_3H. CHI is responsible for the formation of the six-membered heterocyclic ring C of the flavonone which was first isolated by Moustafa and Wong (1967). Three other enzymes then catalyze the conversion of colorless dihydroflavonols to anthocyanins. DFR reduces dihydroflavols to flavan-3,4-ci-diols (leucoanthicyanidins) which then undergo oxidation, dehydration, and glycosylation to form the various anthocyanins, brick-red perlogonidin, red cyanidin, and blue delphinidin pigments (Holton and Cornish, 1995).

The first gene involved in flavonoid biosynthesis was the CHS gene isolated from parsley (Kreuzaler *et al.*, 1983). This led to the isolation of clones of two different CHS genes from petunia (Reif *et al.*, 1985). Of 12 different CHS genes later identified in petunia, however, only four appear to be expressed (Koes *et al.*, 1989). Over the past two decades the transcriptional control of flavonoid biosynthesis has been extensively studied (Broun, 2005). Genetic screens have identified several classes of transcriptional regulators (Rubin *et al.*, 2009). For a more detailed discussion of the regulators of anthocyanin biosynthesis the paper by Gou *et al.* (2011) is recommended.

2. Anthocyanins: Effect of Processing

Anthocyanins are generally unstable during processing with a net loss of color during canning, bottling, and other thermal processing operations. Fruits and vegetables contain many enzymes capable of decolorizing anthocyanins; however, these can be inactivated by blanching. Such enzymes include polyphenol oxidase, anthocyanase, and peroxidase (Grommeck and Markakis, 1964; Peng and Markakis, 1963; Sakamura *et al.*, 1966). In addition to enzymes, thermal stresses, pH, oxygen, and light have all been reported to accelerate anthocyanin degradation (Eskin, 1979; Brouillard, 1983; Hubbermann, 2005; Bordignon *et al.*, 2007; Queiroz *et al.*, 2009; Patras *et al.*, 2010). A recent paper by Hillman *et al.* (2011) showed that the degradation of anthocyanins in grape juice and concentrate increased with increasing temperature and juice concentration. However, of these, pH is the most important factor affecting the stability of the anthocyanin (Mazza and Brouillard, 1987).

Anthocyanins are stable under acidic conditions although model systems show, in most cases, that they are in a colorless form. The ability to retain their color in plants is attributed to the formation of complexes with other



SCHEME 2.17 Anthocyanin and flavonoid biosynthetic pathway (Holton and Cornish, 1995).

phenolics, nucleic acids, sugars, and amino acids, as well as metallic ions such as calcium, magnesium, and potassium (Brouillard, 1983). Several acylated anthocyanins have been identified in flowers which exhibit remarkable stability in neutral or weakly acidic solutions. One such pigment isolated from the petals of Chinese bellflower (*Platycodon grandiflorium*) is platyconin (Saito *et al.*, 1971). Its structure was subsequently confirmed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (Goto *et al.*, 1983). The two acyl groups in this anthocyanin, one located above the pyrilium ring and the other below it, result in its stability in neutral solutions. The presence of two or more acyl residues linked to sugars appears to provide excellent color stability in neutral conditions (Mazza and Brouillard, 1987). Acylated anthocyanins have since been identified in many fruits and vegetables including red onion, red cabbage, red potatoes, broccoli, black carrots, radishes, purple sweet potatoes, and grapes (Giusti and Wrolstadt, 2003; Stintzing and Carle, 2004; Pliszka *et al.*, 2009). Their enhanced stability makes them very attractive as food colorants. Terahara *et al.* (2004) identified four acylated anthocyanins in the callus induced from the storage of purple-fleshed sweet potato (*Ipomoea batatas* L.). They all exhibited higher stability in neutral pH as well as greater DPPH radical scavenging properties compared to the corresponding non-acylated anthocyanins.

The effect of pH on the color of anthocyanins is well established. Harper (1968) showed that perlargonidin chloride was quite stable in a pH range of 1−3 and existed as a deep-red oxonium ion (I). As the pH increased towards neutrality, a loss of red color was observed because of the formation of a colorless pseudobase (II), which was in equilibrium with its keto form (III). The latter then underwent ring opening to the α-diketone form (IV), which was present at pH 3−7. Above pH 7, the anhydro base (V) predominated, producing a purple color which rapidly faded below pH 7 owing to the formation of the pseudobase (II) and α-diketone. Increasing the pH to more alkaline conditions, the purple color changed from mauve to blue because of the formation of the ionized anhydro base (VI). The formation of a brown precipitate was due to degradation of the ketone, which was responsible for the decoloration in anthocyanin-containing fruits during prolonged storage (Scheme 2.18).

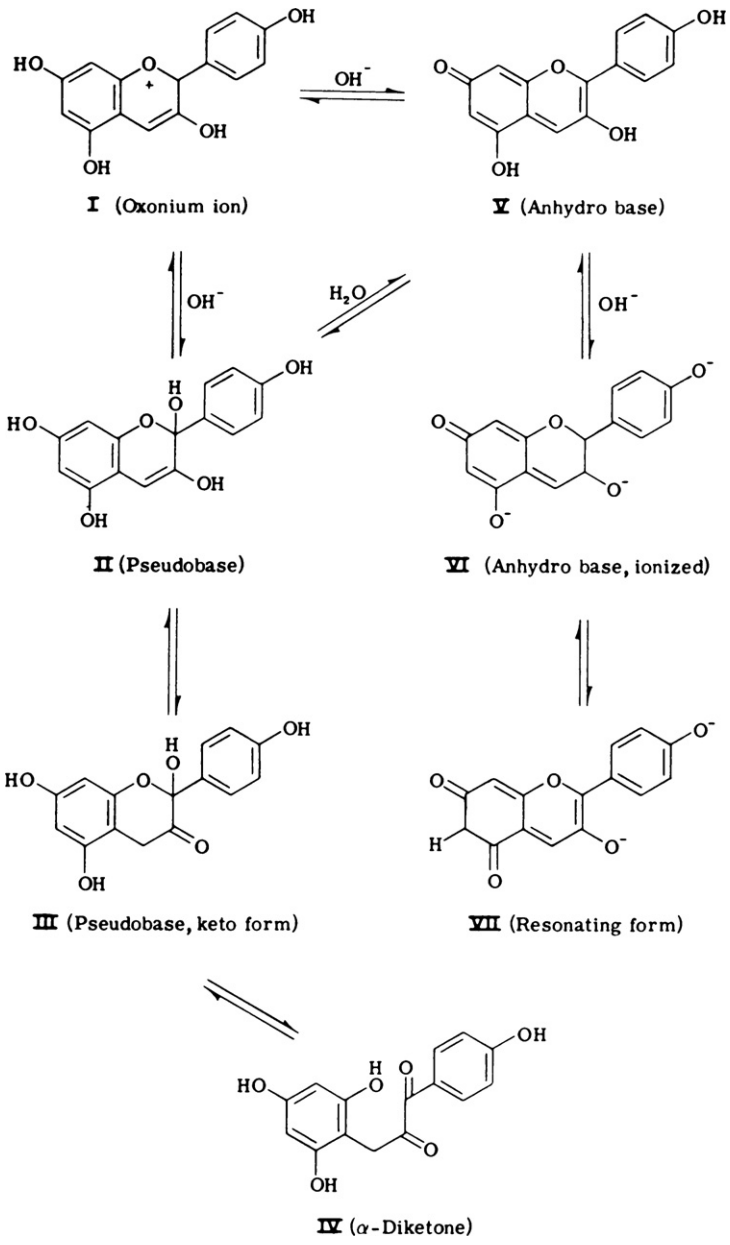
However, Gonnet (1998) showed that spectral variations to describe the color changes in anthocyanin solutions, as affected by pH, should include the entire spectral curve and not just their visible lambda max. The color description should also include tristimulus color attributes of hue, saturation, and lightness. While the CIELAB scale has been reported in a wide range of colors, juices, extracts, and flowers, and some pure anthocyanins, Torskangerpoll and Anderson (2005) noted that no work has been conducted on color variation during the prolonged storage of anthocyanins over the entire pH range. These researchers examined the effect that anthocyanin structure, such as 5-glycosidic substitution and aromatic acylation, has on color stability over 98 days' storage at 10°C over a pH range of 1.1−10.5. Under these conditions, three anthocyanin pigments were examined: cyanidin 3-glucoside, cyanidin-3-(2′-glucosyl-glucoside)-5-glucoside, and cyanidin-3-(2′-(2′-sinapoylglucosyl)-6′-sinapoylglucoside)-5-glucoside. They confirmed the importance of structure in determining the properties of anthocyanins, with the acylated anthocyanins exhibiting greater stability at all pH values except at pH 1.1 and at the more alkaline conditions. At pH 1.1, all pigments retained their color during storage, whereas they all underwent dramatic color changes under the more alkaline conditions.

VI. TEXTURE

The texture of fruits and vegetables is related to the structure and organization of the plant cell walls and intercellular cementing substances (Eskin, 1979). The architecture of the cell wall has been the subject of numerous studies suggesting that it is composed of cellulose fibrils located in a matrix of pectic substances, hemicellulose, proteins, lignin, low-molecular-weight solutes, and water (Van Buren, 1979). In edible plants, the primary cell wall is of central interest as secondary cell walls are virtually absent in mature fruit (Nelmes and Preston, 1968). In fact, the primary cell wall provides most of the dietary fiber in our diet (Jarvis, 2011). During the ripening of fruits, a loss of texture results from the deterioration of the primary cell wall constituents. This is in sharp contrast to the maturation of vegetables, which is accompanied by a toughening of the texture. The latter is due to the development of the secondary cell walls in vegetables, which contribute to the tough and fibrous texture, as a result of lignin deposition.

A. Cell Wall

The cell walls of plants are composed primarily of complex carbohydrates (Northcote, 1963; Jarvis, 2011). The presence of a protein fraction was later established in the primary cell wall of plants (Lamport, 1965). Because of the complexity of the cell wall, numerous models have been proposed, including the first complete model by Keegstra *et al.* (1973). These models tended to raise more questions than answers, however, as none described the cell wall structure adequately. The general approach has been to obtain hydrolyzed fragments, characterize them, and



SCHEME 2.18 Changes in the molecular structure of perlargonidin chloride with pH (Harper, 1968).

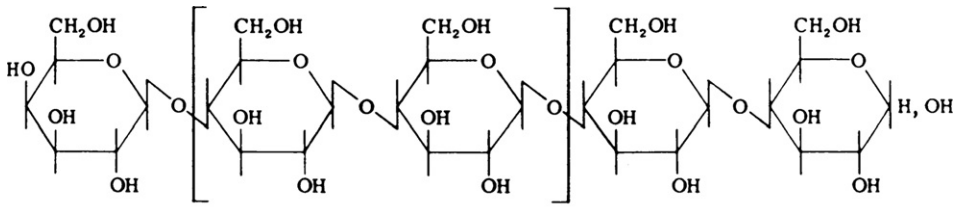
reconstruct the individual fragments. Early research suggested that the hydroxyproline-rich proteins are covalently linked to the pectic substances through the hemicellulose fraction xyloglucan (Hayashi, 1989). Since then, three major families comprising the hydroxyproline-rich glycoproteins (HRGPs) have been identified (Kieliszewski and Shpak, 2001). Textural changes occurring during ripening of fruit are due primarily to enzymatic changes in cell wall architecture. Most affected is the middle lamella, an intercellular cement between the primary cell walls of adjacent cells, which is particularly rich in pectic substances.

1. Cell Wall Constituents

a. Polysaccharides

An important constituent of the primary wall of higher plants is cellulose. It is present as linear aggregates or microfibrils of around 36 β-1,4-glucan chains about 3 nm in diameter (Somerville, 2006). The individual chains are

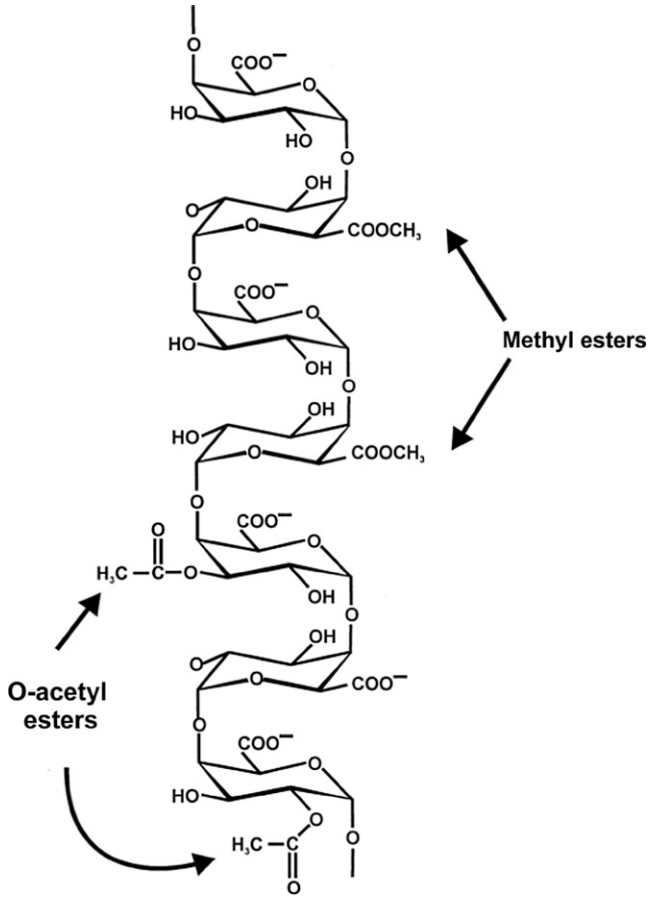
held together by hydrogen bonds through the hydroxyl groups at carbon-6 in one cellulose chain with glycosidic oxygens of the adjacent chains (Northcote, 1972). Within the microfibril are highly organized or crystalline regions as well as some amorphous regions. Cellulose serves as a scaffold that binds other cell wall components (Lerouxel *et al.*, 2006). For a thorough discussion of cellulose structure and biosynthesis, the following reviews are recommended: Doblin *et al.* (2003), Hayashi *et al.*, (2005), Saxena and Brown (2005), and Joshi and Mansfield (2007).



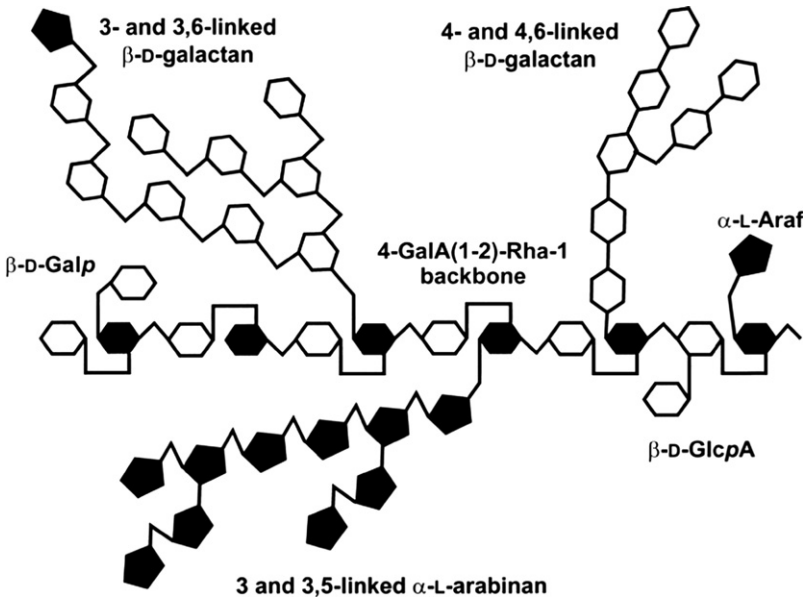
Cellobiose

b. Pectic Substances

Pectic substances comprise one-third of the dry substance of the primary cell walls of fruits and vegetables (Van Buren, 1979). They are complex polysaccharides that include domains of homogalacturonan (HG) and rhamnogalacturonan I (RG-I) with minor amounts of rhamnogalacturonan II (RG-II) (Lerouxel *et al.*, 2006). The basic structure of HG, which accounts for over 60% of the cell wall pectins, consists of a polymer of 1,4-linked α-D-galacturonic acid in which some of the carboxyl groups are methyl esterified (Scheme 2.19) (Ridley *et al.*, 2001). In some plants HG may be partially O-acetylated at O_2 and O_3 (Ishii, 1995, 1997). HG is covalently linked to RG-I



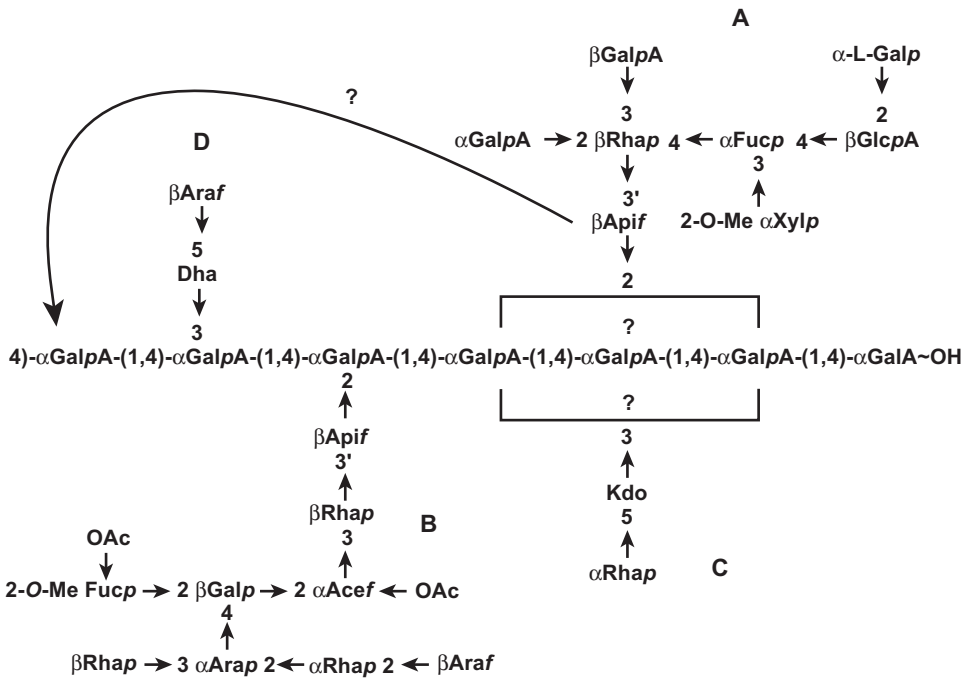
SCHEME 2.19 The primary structure of homogalacturonan (Ridley *et al.*, 2001).



SCHEME 2.20 A model showing the structural features of rhamnogalacturonan I (Ridley *et al.*, 2001).

and RG-II and was hypothesized to be covalently cross-linked to the hemicelluloses fraction, xyloglucan (XG) (Popper and Fry, 2008). The potato cell wall is rich in HG, which is particularly dense in the middle lamellae (Bush, 2001). The HG in the cell walls of tomatoes and mangoes was reported to contain about 35% and 52% uronic acid, respectively (Muda *et al.*, 1995). RG-1 is composed of a backbone of the repeating disaccharide, in which the GalpA residues may be acetylated on C2 and/or C3 (Komalavilas and Mort, 1989) (Scheme 2.20).

RG-II is structurally quite different, with a backbone of [→4)- α-D-GalpA-(1→2)-α-L-Rhap-(1→] (O'Neill *et al.*, 1990) (Scheme 2.21). For a more detailed description of pectins, the reviews by Ridley *et al.* (2001) and Caffall and Mohnen (2009) are recommended.



SCHEME 2.21 The primary structure of rhamnogalacturonan II (Ridley *et al.*, 2001).

c. Hemicellulose

Hemicelluloses, the third and heterogeneous group of polysaccharides in plant cell walls, all have β-(1→4)-linked backbones with an equatorial configuration (Scheller and Ulvskov, 2010). The main groups present in all terrestrial plants include xyloglucan, xylans, mannans, glucomannans, and β-(1→3,1→4)-glucans. At one time galactans, arabinans, and arabinogalactans were also considered to be hemicelluloses. However, Scheller and Ulvskov (2010) pointed out that they appear to be a part of pectin molecules and do not have the equatorial β-(1→4)-linked backbone structure. Xyloglucan, the major hemicellulose in the primary cell walls, is a neutral polysaccharide composed of a β-(1→4)-D-glucose backbone with side-chains rich in α-D-xylose, β-D-gulactose and occasionally some α-fucose residues (Popper and Fry, 2008). For a more detailed discussion of hemicelluloses the review article by Scheller and Uvskov (2010) is strongly recommended.

d. Proteins

Proteins identified in the primary cell walls of plants include those rich in hydroxyproline. This family of hydroxyproline-rich glycoproteins (HRGPs) includes three major groups, the highly periodic and lightly arabinosylated repetitive proline-rich proteins (PRPs), the periodic and highly arabinosylated extensins, and the most highly glycosylated and least periodic arabinogalactan-proteins (Kieliszewski and Shpak, 2001). In addition to extensin and hydroxyl-proline-rich proteins, a third group of proteins has been identified in plant cell walls. These are glycine-rich proteins which are also part of structural components of the plant cell wall (Ringli *et al.*, 2001). A proteomic approach to plant cell wall proteins was presented by Jamet *et al.* (2006).

e. Lignin

Lignin includes a large group of aromatic polymers produced from the oxidative coupling of 4-hydroxyphenylpropanoids (C_6—C_3) (Boerjan *et al.*, 2003; Ralph *et al.*, 2004). These polymers are primarily deposited in the secondary cell walls and provide mechanical strength to the plant cell walls. They are also responsible for the toughness of such vegetables as celery and asparagus (Segerlind and Herner, 1972; Herner, 1973). For a more detailed discussion of lignin a recent review by Vanholme *et al.* (2010) is recommended.

f. Cell Wall Biosynthesis

Considerable progress has been made over the past two decades on the biosynthesis of the complex polysaccharides and their deposition in the cell wall (Lerouxel *et al.*, 2006; Joshi and Mansfield, 2007; Caffall and Mohnen, 2009). While there are still gaps in our knowledge, we have a much better understanding of its regulation (Zhong and Ye, 2007).

Pectin biosynthesis occurs in the Golgi apparatus, a complex organelle composed of stacks of flattened vesicles containing proteins. The current model for pectin biosynthesis suggests that nucleotide sugars are synthesized on the cytosolic side of the Golgi and transported into the Golgi lumen by membrane-spanning protein transporters, or alternatively synthesized in the Golgi lumen (Scheller *et al.*, 1999; Scheible and Pauly, 2004; Mohnen, 2008; Mohnen *et al.*, 2008). Multiple activities are required for the complete biosynthesis of HG, RG-I and RG-II, with many glucosyltransferases (GT) involved (Scheller *et al.*, 1999; Moyne *et al.*, 1999, 2008). For example, the complete synthesis of HG requires several specific catalysts, α1,4-GalATs (HG:GalATs), to transfer D-GalA from UDP-D-GalA to the growing HG polymer (Caffall and Mohnen, 2010). HG:GalAT has since been characterized in tomato and turnip (Lin *et al.*, 1966; Bolwell and Northcote, 1983), peas (Sterling *et al.*, 2001), and pumpkin (Ishii, 2002).

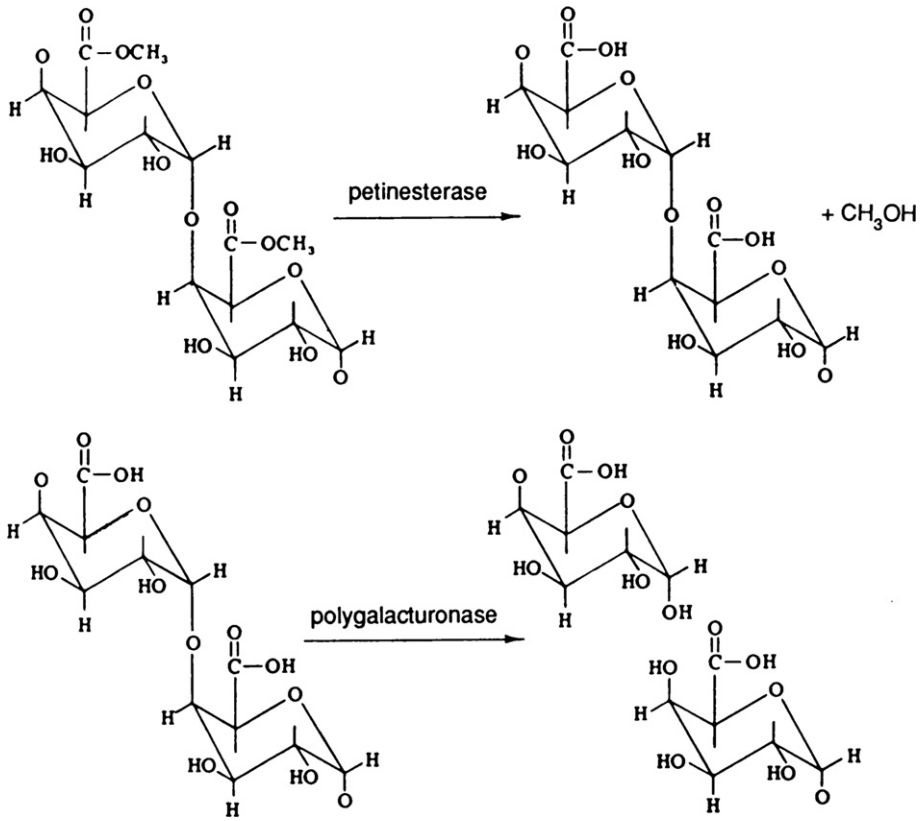
Once formed, GHHG can be modified by the addition of methyl groups at the C6 carboxyl group or acetyl groups at O2 and O3 of the GalA residues (Willats *et al.*, 2001). Pectin methyltransferases (PMTs) then act specifically on the pectic polysaccharides in the Golgi apparatus by transferring a methyl group from the donor *S*-adenosyl-methionine (SAM) (Villemez *et al.*, 1966; Kauss *et al.*, 1967; Bruyant-Vannier *et al.*, 1996), releasing them in a highly methylesterified form (Li *et al.*, 1997; Lennon and Lord, 2001). Multiple PMT enzymes are needed for the synthesis of pectin, as different isoforms were discovered each exhibiting their own pH optima and substrate preference (Krupkova *et al.*, 2007). For a discussion of the biosynthesis of RG-I and RG-II, the article by Caffall and Mohnen (2009) is recommended.

The biosynthesis of a cellulose-like (1→4) glucan involves several cytosolic and membrane-bound enzymes that primarily produce pools of UDP-glucose. The latter provides the primary substrate for cellulose synthase (Carpita and McCann, 2000). Two pathways were found to lead directly to the production of UDP-glucose (Joshi and Mansfield, 2007). In the first pathway, sucrose is cleaved by sucrose synthase, releasing UDP-glucose and fructose. The second pathway is much more dependent on the hexose phosphate pool, with UDP-glucose pyrophosphorylase (UGPase) phosphorylating

glucose-1-phosphate. An alternative, and indirect pathway for producing UDP-glucose, is through the hydrolysis of sucrose by invertase. Irrespective of how UDP-glucose is produced, it is then catalyzed by glycosyltransferase to form the cellulose polymer. This enzyme is referred to as inverting, as the glucosyl residue in UDP-glucose is in the α-configuration which must be converted to the β-linkage, exclusive to cellulose (Guerriero *et al.*, 2010). A major breakthrough in understanding cell wall biosynthesis was identification of genes encoding cellulose synthase (C*es*) in cotton fiber (Pear *et al.*, 1996; Delmer, 1999). Cellulase synthase (C*es*) has since been identified in other higher plants. For a more detailed review of cellulose biosynthesis including the formation of microfibrils, the following reviews are recommended: Lerouxel *et al.* (2006), Joshi and Mansfield (2007), and Guerriero *et al.* (2010).

2. Cell Wall Degradation

The softening of fruits during ripening is attributed to changes in pectin (Kertesz, 1951; Hobson, 1967; Tavakoli and Wiley, 1968; Pressey *et al.*, 1971). This is characterized by a decrease in the level of insoluble pectin (protopectin) with a concomitant increase in soluble pectic substances (Ben-Arie *et al.*, 1979). Protopectin is the generic name ascribed to the water-insoluble high-molecular-weight parent pectin substances present in unripe fruit. Little is known about this polymer other than that its hydrolysis during ripening yields soluble polyuronides, pectin, and pectinic acids (Worth, 1967; Eskin, 1979; John and Dey, 1986). The latter are polygalacturonic acid polymers varying in the degree of methylation. The pectic material in the middle lamella is quite different from that found in the primary cell walls and consists of pectic substances in the free state or as the corresponding calcium pectate (Eskin, 1979). The degradation of the loosely bound soluble pectin is responsible for the softening of the texture during ripening (Doreyappa *et al.*, 2001). These pectin-degrading enzymes are classified according to their mode of action on pectin and pectic substances and include polygalacturonases (PG) (EC 3.2.1.15), pectin methylesterases (PM) (EC 3.1.1.11), and pectate and pectin lyases (PL) (EC 4.2.2.2) (Sakai *et al.*, 1993; Wong, 1995; Chauhan, 2001).



a. Polygalacturonases

Polygalacturonase (PG), an important pectolytic glycanase, has been primarily implicated in the softening of fruit during ripening (Poovaiah and Nukuya, 1979). It is considered to be primarily responsible for the dissolution of the

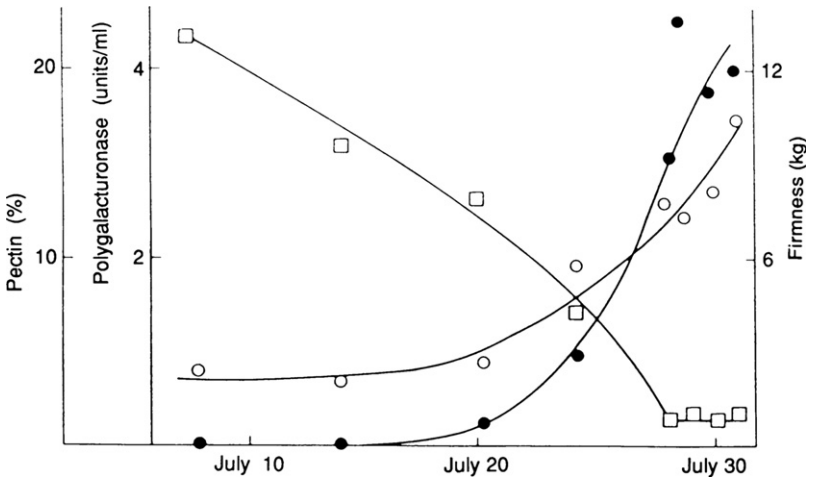
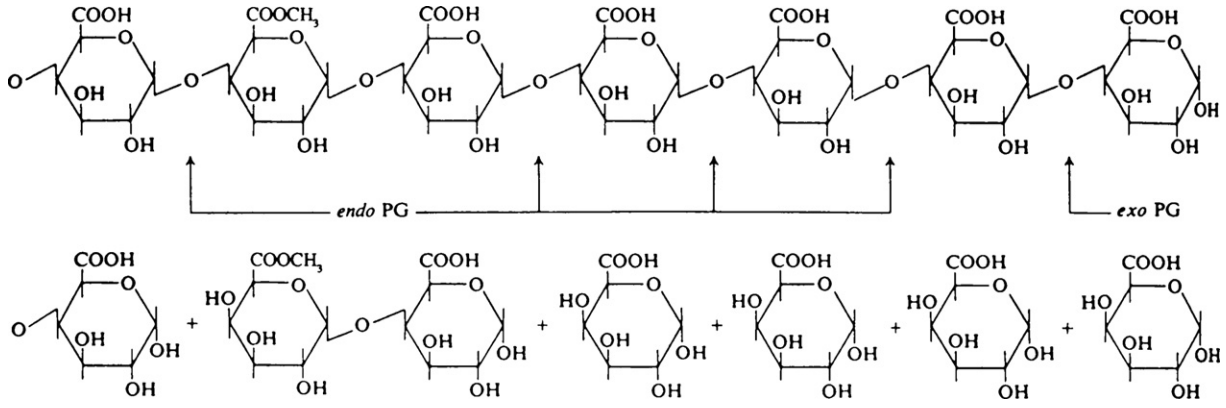


FIGURE 2.10 Changes in firmness (□), PGA activity (●), and water-soluble pectin (○) in Elberta peaches during ripening (Pressey *et al.*, 1971). *(Copyright © by Institute of Food Technologists.)*

middle lamella during ripening (Jackman and Stanley, 1995; Voragen *et al.*, 1995). Since the preferred substrate of these enzymes is D-galacturonans, Rexova-Benkova and Markovic (1976) referred to them as D-galacturonases. These enzymes have been reported in many fruits, including peaches (Pressey and Avants, 1973a), pears (McCready and McComb, 1954; Pressey and Avants, 1976; Bartley and Knee, 1982), and tomatoes (Foda, 1957; Patel and Phaff, 1960a, b; Hobson, 1964). The activity of these enzymes increases during the ripening process, when it hydrolyzes pectic material in the middle lamellae and cell walls (Hobson, 1965; Pressey, 1977). The change in polygalacturonase activity during ripening is illustrated in Figure 2.10 for peaches. Pressey *et al.* (1971) found that the increase in enzyme activity was accompanied by an increase in water-soluble pectin and fruit softening.

Two types of polygalacturonases or D-galacturonases have been identified, endo and exo. The former randomly hydrolyzes the glycosidic bonds in the pectin molecule while the exo-enzyme acts from the terminal end of the pectin molecule (Scheme 2.22). In the presence of endopolygalacturonases, the pectin molecules are rapidly degraded into smaller units, accompanied by a marked decrease in viscosity. Both forms of the enzyme are found in pears (Pressey and Avants, 1976; Bartley and Knee, 1982) and peaches (Pressey and Avants, 1978). However, some fruit species lack any detectable endo-PG, including apples (Brackmann *et al.*, 1996; Goulao *et al.*, 2007), strawberries (Abeles and Takeda, 1990), and grapes (Nunan *et al.*, 2001). The greater degree of softening reported by Pressey and Avants (1978) for 'freestone' peaches compared to 'clingstone' peaches was attributed to the absence of endopolygalacturonase in the clingstone fruit. The random degradation of pectin by endopolygalacturonase together with exopolygalacturonase rapidly solubilized the pectin in freestone peaches. The absence of endopolygalacturonase in clingstone peaches was evident by the greater retention of protopectin during ripening.

Exopolygalacturonases (EC 3.2.1.67) have been identified in peaches (Pressey and Avants, 1973a), pears (Pressey and Avants, 1976), cucumbers (Pressey and Avants, 1975; McFeeters *et al.*, 1980), and bananas (Markovic *et al.*,



SCHEME 2.22 Action of exo- and endo-polygalacturonases.

1975). Exopolygalacturonase is the only D-galacturonase found in apples and is responsible for the release of galacturonic acid and polyuronides (Bartley, 1978). Studies using cell wall isolates from tomatoes (Wallner and Bloom, 1977; Gross and Wallner, 1979; Themmen *et al.*, 1982) and pears (Ahmed and Labavitch, 1980) demonstrated that endopolygalacturonases played the major role in pectin degradation during ripening. The release of a water-soluble polymer (WSP) of molecular weight 20,000 containing galacturonic acid and rhamnose by endopolygalacturonase from the cell walls of red tomatoes was reported by Gross and Wallner (1979) to be identical with the polymer released by the same enzyme from the cell walls of mature green tomatoes. While the activity of polygalacturonases in ripe red tomatoes is predominantly the endo form, it is the exo form that is mainly present in the corresponding green fruit (Pressey and Avants, 1973b; Tucker *et al.*, 1980). Although exopolygalacturonase represents only a small fraction of polygalacturonase activity, it was present throughout the ripening of tomatoes at fairly constant levels. Pressey (1987) suggested, therefore, that it was unlikely that exopolygalacturonase played an important part in pectin degradation, but they might have some role in the growth and development of the tomato fruit.

Isoenzyme forms of PG have been reported in a wide range of fruits including bananas (Pathak and Sanwal, 1998), strawberries (Nogota *et al.*, 1993), and peaches (Pressey and Avants, 1973a). Two isoforms of endo-PG, PG_1 and PG_2, were reported in tomatoes (Pressey and Avants, 1973b). Subsequent studies by Tucker *et al.* (1980) suggested that PG_1 was a dimer of PG_2. Further work by Pressey (1986a, b) suggested that PG_1 was a combination of PG_2 and a β-subunit.

b. Pectin Methyl Esterase: Pectinesterase

Pectin methyl esterases (PMs), or pectinesterases, are widely distributed in many fruits, including bananas (Buescher and Tigchelaar, 1975), peaches (Nagel and Patterson, 1967), and strawberries (Barnes and Patchett, 1976). Considerable confusion surrounds the early studies on PM during ripening. For example, Hultin and Levine (1965) noted a rise in PM activity during the ripening of bananas which was not observed by De Swardt and Maxie (1967) when they used polyvinylpyrrolidine (PVP) to remove polyphenols. Brady (1976) also found very little change in PM activity in banana extracts following the addition of 2-mercaptobenzothiazole, a potent inhibitor of banana polyphenol oxidase. PVP was later found by Awad and Young (1980) to suppress PM activity, although no inhibitory effects were exerted by the endogenous phenols. PM does not appear to have a major role in fruit softening, as it is present at high levels in underdeveloped fruit, such as tomatoes and bananas, prior to ripening (Barnes and Patchett, 1976; Brady, 1976; Pressey and Avants, 1982a; Tucker *et al.*, 1982). In the case of avocados, however, there is a dramatic drop in PME activity just before ripening, which was reported to be a useful index of fruit maturity (Zauberman and Schiffman-Nadel, 1972). A decrease in PM activity in avocados by as much as 50% prior to the climacteric proved to be a reliable indicator of softening time when stored in controlled atmospheres (Barmore and Rouse, 1976). A drop of 80% in PME activity was also reported in avocados by Awad and Young (1980).

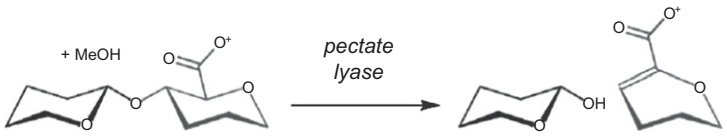
The traditional view of PM is of de-esterification of the cell wall galacturonans followed by polygalacturonase action. This de-esterification of galacturonans was found by several researchers (Dahodwala *et al.*, 1974; Rexova-Benkova and Markovic, 1976) to enhance the activities of both endo- and exopolygalacturonases. Stimulation of tomato endopolygalacturonases by PM was reported by Pressey and Avants (1982b), although these enzymes were capable of hydrolyzing highly esterified substrates at pH 3.5. The presence of a high degree of pectin methylation during the ripening of avocados (Dolendo *et al.*, 1966), apples (Knee, 1978), and peaches (Shewfelt *et al.*, 1971) pointed to a rather limited role for PM in fruit softening. Ben-Arie and Sonego (1980) attributed the development of woolly breakdown of peach flesh during cold storage to the inhibition of polygalacturonase activity and enhancement of PM. This phenomenon was attributed to the inability of peaches to undergo the desirable textural changes associated with normal development. Recent studies by von Mollendorff and De Villiers (1988), however, showed that the primary cause of woolliness in peaches was the sudden rise in the level of polygalacturonase, while the role of PME was far less clear. Nevertheless, the cause of woolliness in peaches remains confusing as some studies have associated woolliness in peaches with an increase in PM activity (Brummell *et al.*, 2004; Girardi *et al.*, 2005), a decrease in PM activity (Buescher and Furmanski, 1978; Ben-Arie and Sonego, 1980), or a lack of expression of PM (Obehland and Carroll, 2000; Zhou *et al.*, 2000).

It is evident from these discussions that fruit softening is due to compositional changes in the cell walls of fruit mediated by the combined activity of polygalacturonases and PME. This results in the release of soluble polyuronide with a corresponding decrease in the molecular weight of the polyuronide polymer (Gross and Wallner, 1979; Huber, 1983; Seymour *et al.*, 1987a). The action of polygalacturonase, as discussed previously, may be limited to the

demethylated regions of the polygalacturonan, which is brought about by the action of PM. The enhanced production of polyuronides was reported by Pressey and Avants (1982b) in isolated cell walls of mature green tomato fruit in the presence of PME. Seymour *et al.* (1987a), using enzyme-inactivated cell wall preparations to eliminate the effect of any endogenous enzymes, found that polyuronide breakdown was much lower in *in vivo* than in *in vitro* studies. The fact that pectin was not completely de-esterified, in spite of high levels of PM, suggested that this enzyme may be restricted *in vivo*. Further studies by Seymour *et al.* (1987b) on tomatoes also demonstrated the lower solubilization of polyuronides *in vivo*, which was attributed to the restriction of PM action. The release of two discrete-sized oligomers, together with galacturonic acid, suggested that the combined pectolytic action was not completely random. These researchers indicated the importance of identifying these oligomers *in vivo* in light of the recent discovery of cell wall elicitors.

c. Pectic Lyase

Pectic lyase (PL) [poly(1,4-α-D galacturonide) lyase, EC 4.2.2.1] cleaves the de-esterified or esterified galacturonate units of pectin by trans-β-elimination of the hydrogen on the C4 and C5 positions of galacturonic acid (Prasanna *et al.*, 2007). While there are exo and endo forms of PL, most of those studied are endo type, which act randomly (Wong, 1995).



Adapted from Gilbert *et al.* (2008)

A pectate lyase gene was isolated from ripe strawberries and proposed as a candidate for pectin degradation and loss of fruit firmness (Medina-Escobar *et al.*, 1997). Using transgenic strawberries, Jimenez-Bermudez *et al.* (2002) manipulated softening by antisense expression of a pectate lyase gene. Their results indicated that the rapid softening of strawberries could be slowed down by the pectate lyase gene. More recent research by Payasi and Sanwal (2003) demonstrated pectate lyase activity in ripening bananas. Further purification of pectate lyase by Payasi *et al.* (2008) showed that it required Mg^{2+}, in addition to Ca^{2+}, for maximum activity.

d. Cellulase

Cellulose degradation also occurs during the ripening of tomatoes (Babbitt *et al.*, 1973; Pharr and Dickinson, 1973; Sobotka and Stelzig, 1974), strawberries (Barnes and Patchett, 1976), avocados (Pesis *et al.*, 1978; Awad and Young, 1980), and Japanese pear fruit (Yamaki and Kakiuchi, 1979). The enzyme involved, cellulase, is composed of several distinct enzymes referred to as the 'cellulase complex' (King and Vessal, 1969). These include C_1-cellulase, C_x-cellulase, cellobiase, and exocellulase, which together catalyze the degradation of cellulose as follows:

Insoluble cellulose
↓ C_1-cellulase
Soluble cellulose derivatives
↓ C_x-cellulase
Cellobiose
↓ cellobiase (β-1,4-glucosidase)
Glucose

The degradation of insoluble cellulose to soluble derivatives is poorly understood but appears to involve C_1-cellulase. The breakdown of soluble cellulose is mediated by C_x-cellulase, also referred to by its systematic name 1,4-glucan-4-glucanohydrolase, which randomly cleaves the internal linkages in the cellulose chain. Conflicting reports in the literature suggest that cellulase activity was absent or present at very low levels in unripe fruit, whereas others found cellulase activity in immature tomato fruit (Hobson, 1968; Babbitt *et al.*, 1973). Information on the cellulase complex was derived almost exclusively from studies on microbial cellulase. The first study to identify a similar complex in plants was by Sobotka and Stelzig (1974), who partially purified four cellulase fractions from tomato using ammonium sulfate fractionation. These researchers identified C_1-cellulase, C_1-cellulase, β-glucosidase,

and cellobiase as the first cellulase complex capable of completely degrading insoluble cellulose in plants. Pharr and Dickinson (1973) were unable to identify an enzyme in tomato fruit that could degrade insoluble cellulose, although they did report the presence of C_x-cellulase (EC 3.2.1.4) and cellobiase (EC 3.2.1.21). The presence of a cellulase complex in plants still remains to be established.

The role of cellulase in fruit softening is still somewhat speculative. The only direct evidence with respect to the involvement of cellulase was that reported by Babbitt *et al.* (1973). These researchers investigated the effect of the growth regulators ethephon and gibberellic acid on cellulase and polygalacturonase activities in ripening tomatoes. In the presence of ethephon, cellulase activity increased initially and then declined after 6 days, while polygalacturonase activity increased (Figure 2.11). This contrasted with the almost complete inhibition of polygalacturonase activity by gibberellic acid while cellulase activity continued to increase. The decrease in overall firmness of the tomato fruit in the presence of gibberellic acid pointed to a definite role for cellulase in fruit softening. These researchers proposed that cellulase initiated fruit softening by degrading the cellulose fibrils in the cell walls, which permitted pectic enzymes to penetrate the middle lamella. This could explain the observation by Awad and Young (1979), who found

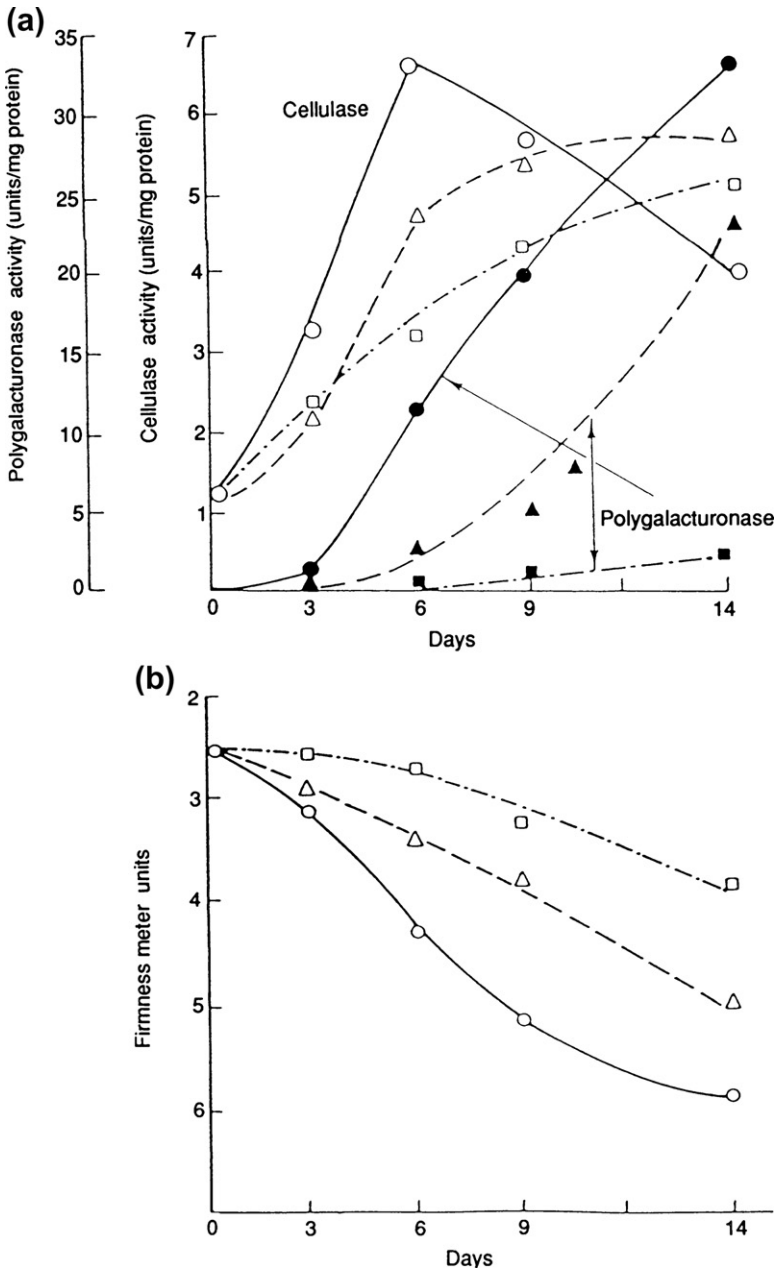


FIGURE 2.11 Effect of ethephon (○) and gibberellic acid (□) on enzyme activity (a) and firmness (b) of tomatoes (△, control) (Babbitt *et al.*, 1973).

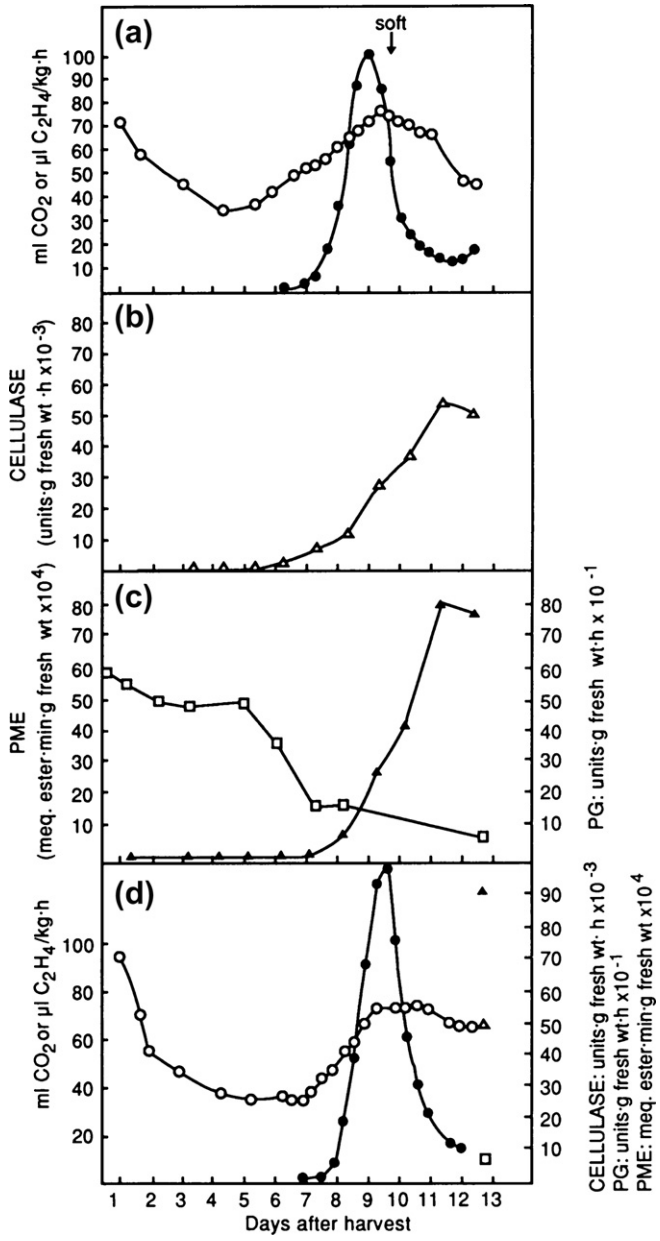


FIGURE 2.12 Postharvest trends in cellulase (Δ), PG (▲), and PME (□) activity and in CO_2 (○) and C_2H_4 (●) production in an individual Fuerte avocado fruit. Fruit in (a) was edibly soft after 9.5 days and in (d) after 10.5 days (Awad and Young, 1979).

that an increase in cellulase activity preceded increases in poylgalacturonase and ethylene production in ripening avocado fruit and subsequent softening (Figure 2.12).

Using isoelectric focusing (IEF), Kanellis and Kalaitzis (1992) isolated multiple active forms of cellulase in the mesocarp of ripe avocados. The cellulases were separated into 11 distinct forms capable of degrading carboxymethylcellulose (CMC) on CMC-agarose gels. Sexton *et al.* (1997) localized cellulase in the regions of red raspberry fruit associated with abscission, suggesting that it may be involved in fruit separation as well as softening.

e. β-Galactosidase

The loss of galactose from the cell walls of apples, strawberries, and tomatoes during ripening is the result of the action by β-galactosidase (EC 3.2.1.23). The increased activity of this enzyme was correlated with the loss of firmness during the ripening and storage of apples (Bartley, 1974, 1978; Wallner, 1978; Berard *et al.*, 1982). Evidence for this is provided by a decrease in the galactose content of apple cortex cell walls (Knee, 1973), an increase in the

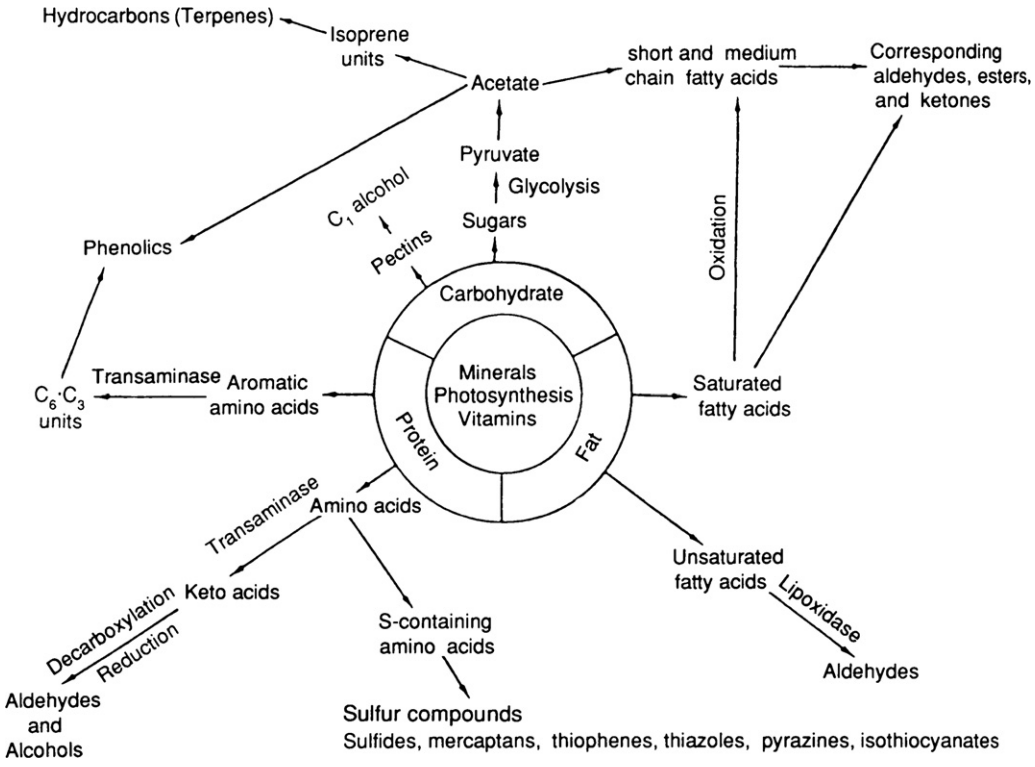
soluble polyuronide content (Knee, 1975), the ability of β-galactosidase to break down β-(1→4)-linked galactan (Bartley, 1974), and the release of galactose from cell wall preparations (Bartley, 1978). Dick *et al.* (1984) provided preliminary evidence for the regulation of β-galactosidase activity in 'McIntosh' apples by the presence of an endogenous inhibitor. Unlike β-galactosidase in apples, that found in tomatoes did not appear to be involved in cell wall hydrolysis of galactans (Gross and Wallner, 1979). In fact, these researchers were unable to detect any β-galactosidase activity during the ripening of tomatoes. This contrasted with a later study by Pressey (1983), who isolated three enzymes responsible for β-galactosidase activity in tomatoes. One of these enzymes hydrolyzed tomato galactans and increased in activity during the ripening process. This suggested a possible role for β-galactosidase in fruit softening. Since the galactan polysaccharide in tomato fruit is (1→4)-linked, the β-galactosidase involved must be β-1,4-galactosidase. The inability of Gross and Wallner (1979) to detect any galactanase activity was attributed to the preparation of their extracts from frozen fruit. As pointed out by Pressey (1983), the yield of β-galactosidase in frozen fruit is very low compared to that in the corresponding fresh tomato extracts. Ranwala *et al.* (1992) monitored the changes in the soluble and wall-bound β-galactosidases during the ripening of muskmelon fruit. They demonstrated the involvement of β-galactosidases in modifying the pectic and hemicelluloses components, indicating a possible role in the softening of muskmelon.

VII. FLAVOR

The flavor of fruits and vegetables is a complex interaction between aroma and taste. Aroma is produced by the volatiles synthesized during fruit ripening, which include aldehydes, ketones, alcohols, esters, lactones, terpenes, and sulfur compounds (Baldwin, 2004; Pérez and Sanz, 2008). Vegetables, with a few exceptions, tend to be more bland in flavor. Their aroma is determined by secondary metabolites such as terpenes, phenolics, glucosinolates, and cysteine sulfoxides (Jones, 2008). Taste is provided by many non-volatile components, including mainly sugars and acids, as well as compounds with bitter taste or astringency present in the fruit flesh or vegetable tissue.

A. Aroma

Volatiles responsible for aroma originate from proteins, carbohydrates, lipids, and vitamins, as shown in Scheme 2.23. The aroma characteristics of individual fruit and vegetable crops are determined by genetics as well as



SCHEME 2.23 Formation of volatile aroma in fruits and vegetables. (Reprinted from Salunkhe *et al.*, 1976, with permission.)

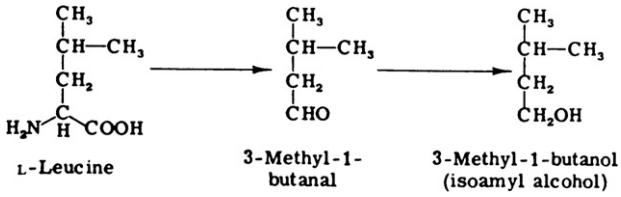
preharvest, harvesting, and postharvest factors, and usually develop during ripening and maturation (Kader, 2008). Many volatiles have been identified in the literature and were reviewed by Salunkhe and Do as early as 1976. Since then, studies on a wide range of produce have been undertaken. A well-researched fruit is the apple, in which the impact of preharvest and postharvest factors has been extensively studied. Over 300 volatile compounds are produced by apple fruits; the most abundant group comprises esters (Yahia, 1994; Dixon and Hewett, 2000). Over 400 volatile compounds are generated by tomato fruit (Hobson and Grierson, 1993) and differences in flavor between varieties are due to variation in aroma volatile production (Brauss *et al.*, 1998). However, qualitative and quantitative differences between research groups may be caused by the use of a vast range of extraction and analytical methods (Saevels *et al.*, 2004). Furthermore, instrumental analysis of volatiles must be combined with sensory analysis to provide insight into the impact of volatile compounds on flavor perception (Baldwin *et al.*, 2007; Song, 2007). A new approach to elucidate responses of human odorant receptors to aroma compounds includes molecular sensory science (Greger and Schieberle, 2007; Schmiech *et al.*, 2008). In view of optimizing and improving the flavor quality of fruits and vegetables and gaining a better understanding of flavor volatile production and regulation, current research has been directed towards the isolation of genes encoding key enzymes involved in the biosynthesis of aroma volatiles (Song, 2007; Pech *et al.*, 2008) based on research in recent decades focusing on volatile identification and biosynthesis. Control of formation of volatiles depends on many factors such as the genes involved and hence on their expression and transcription, the functionality of enzymes catalyzing modifications of substrates to volatiles, and the availability of substrates or precursors, as well as the energy status of cells (Saquet *et al.*, 2003b; Souleyre *et al.*, 2005; Song, 2007; Schwab, 2008; Wyllie, 2008). This section will focus briefly on a few of the biogenic pathways involved in volatile formation during ripening.

1. Aldehydes, Alcohols, and Esters

Short-chain unsaturated aldehydes and alcohols and straight chain and branched chain esters are important contributors to the aroma volatiles of fruits. These are formed during the short ripening period associated with the climacteric rise in respiration (Paillard, 1968; Romani and Ku, 1968; Tressl *et al.*, 1970; Song and Bangerth, 2003; Lara *et al.*, 2003; Souleyre *et al.*, 2005). Studies on apples, bananas, pears, and tomatoes have shown them to be synthesized from amino acids or fatty acids (Eskin *et al.*, 1977; Eskin, 1979; Song and Bangerth, 2003; Rapparini and Predieri, 2003).

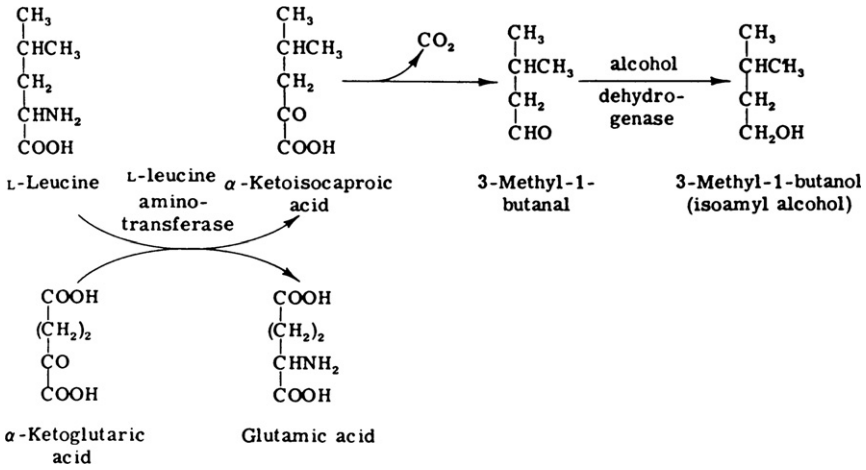
a. Amino Acids as Precursors

An increase in 3-methyl-1-butanol, isopentyl acetate, isopentyl butyrate, and isovalerate volatiles was reported by Dalal (1965) during tomato ripening. At the same time, 3-methyl-1-butanal increased up to the breaker stage and then decreased. The similarity between the alcohol portion of these esters and the carbon skeleton of 3-methyl-1-butanal suggested to Yu *et al.* (1968a) that they were synthesized from this aldehyde. Since leucine had an identical carbon skeleton to that of 3-methyl-1-butanal, the possible role of this amino acid in the synthesis of this aldehyde was investigated by Yu *et al.* (1968c). On the basis of their work with L-[^{14}C]leucine, the following pathway was proposed:



In addition to leucine, aspartic acid, alanine, and valine were shown to produce carbonyl compounds when added to tomato extracts (Yu *et al.*, 1968c; Buttery and Ling, 1993). The decrease in the levels of these amino acids during ripening pointed to the presence of enzymes capable of utilizing them. The soluble fraction obtained by centrifugation of tomato extracts was particularly active on leucine, while aspartic acid and alanine were metabolized by the mitochondrial fraction. Based on the detection of large amounts of glutamic acid in tomato extracts, Freeman and Woodbridge (1960) and Yu *et al.* (1967) both pointed to the presence of active transaminases. This was confirmed in a subsequent study by Yu *et al.* (1968b), who found a marked production of glutamic acid when extracts of field-grown tomatoes at the green and ripe stages were incubated with these amino acids. Yu and Spencer (1969) incubated L-leucine with fresh tomato extracts and isolated α-keto-isocaproic acid among the products formed. Confirmation

with labeled [^{14}C]leucine established the presence of L-leucine:2 keto-glutarate amino transferase in tomatoes. Scheme 2.24 summarizes the reactions leading to 3-methyl-1-butanol from L-leucine. Studies with deuterium-labeled substrates in apples corroborated these findings (Rowan *et al.*, 1996, 1999). Finally, aromatic amino acids can be converted to esters through the same pathways in different fruits (Tressl and Albrecht, 1986; Tikunov *et al.*, 2005).



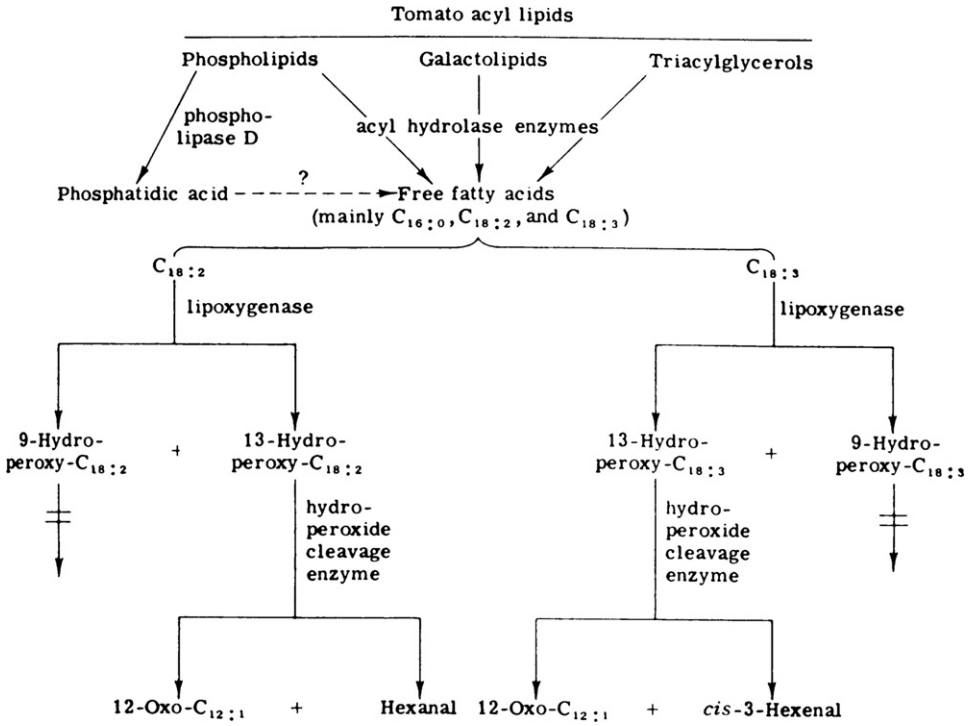
SCHEME 2.24 Biogenesis of isoamyl alcohol from L-leucine in tomato fruit.

b. Fatty Acids as Precursors

The volatile carbonyls responsible for the aroma of tomatoes and bananas as well as other commodities are synthesized from unsaturated fatty acids (Goldstein and Wick, 1969; Jadhav *et al.*, 1972; Song and Bangerth, 2003). β-Oxidation and the lipoxygenase pathway are the main metabolic pathways generating aroma in fruits (Baker *et al.*, 2006). The major unsaturated fatty acids in the pericarp of tomatoes were shown to be oleic, linoleic, and linolenic acids (Kapp, 1966). Jadhav *et al.* (1972) reported a decrease in the levels of both linoleic and linolenic acids as the tomato fruit ripened. A marked decrease in linoleic acid was also observed by Goldstein and Wick (1969) in ripe banana pulp, which suggests a possible relationship between this fatty acid and the production of volatiles. Similarly, Song and Bangerth (2003) reported that fatty acids served as precursors of aroma volatiles in preclimacteric and climacteric apple fruits.

Incorporation of ^{14}C-labeled linoleic and linolenic acids into hexanal using tissue slices or cell-free tomato extracts suggested to Jadhav *et al.* (1972) the involvement of lipoxygenase. This was confirmed by the total inhibition of carbonyls in the presence of hydrogen peroxide, a recognized inhibitor of lipoxygenase. Consequently, this enzyme was monitored during tomato ripening and found to increase in activity at the onset of the climacteric. This appeared to explain the increase in volatiles that accompanied maturation of the tomato fruit (Dalal *et al.*, 1968). Kazeniac and Hall (1970) reported the presence of higher levels *cis*-3-hexenal, *trans*-2-hexenal, and *n*-hexanol in fully ripened tomato fruit. The formation of *trans*-2-hexenal resulted from the instability of *cis*-3-hexenal to the acidic pulp and juice of the tomato, with isomerization to the *trans* isomer. Stone *et al.* (1975) showed that *cis*-3-hexenal was the major volatile of tomato distillates, while Jadhav *et al.* (1972) found *n*-hexanol to be the major volatile formed. This discrepancy suggested to Stone *et al.* (1975) that *cis*-3-hexenal was a precursor of *n*-hexanol, but this was discounted when only 2% of *cis*-3-[^{14}C]hexenal was incorporated into the alcohol form. Jadhav *et al.* (1972) attributed the presence of hexanol, propanol, 2,4-decadienal, 2,6-heptadiene, and *cis*-3-hexenal in tomato volatiles to the formation of 9-, 12-, and 16-hydroperoxides by the action of lipoxygenase on linoleic and linolenic acids. Gaillard and Matthew (1977), however, reported that the major fatty acid hydroperoxides formed from linoleic and linolenic acids were 9- and 13-hydroperoxides in a ratio of 95:5. Of these, only the 13-hydroperoxide was cleaved to form the non-volatile compound 12-oxo-dodec-*cis*-9-enoic acid, together with hexanal and *cis*-3-hexenal from linoleic and linolenic acids, respectively (Scheme 2.25). Zamora *et al.* (1987) characterized lipoxygenase from tomato fruit and confirmed the 9-hydroperoxide isomer to be the major one formed from linoleic acid. The ratio of 9- to 13-hydroperoxide isomers produced from linoleic acid was found to be 24:1, in close agreement with that reported previously by Gaillard and Matthew (1977).

Buttery and co-workers (1987) developed improved trapping methods for the quantitative analysis of the major C_4–C_6 volatiles in tomato fruit. In addition to inactivating tomato enzymes which affected the volatiles during

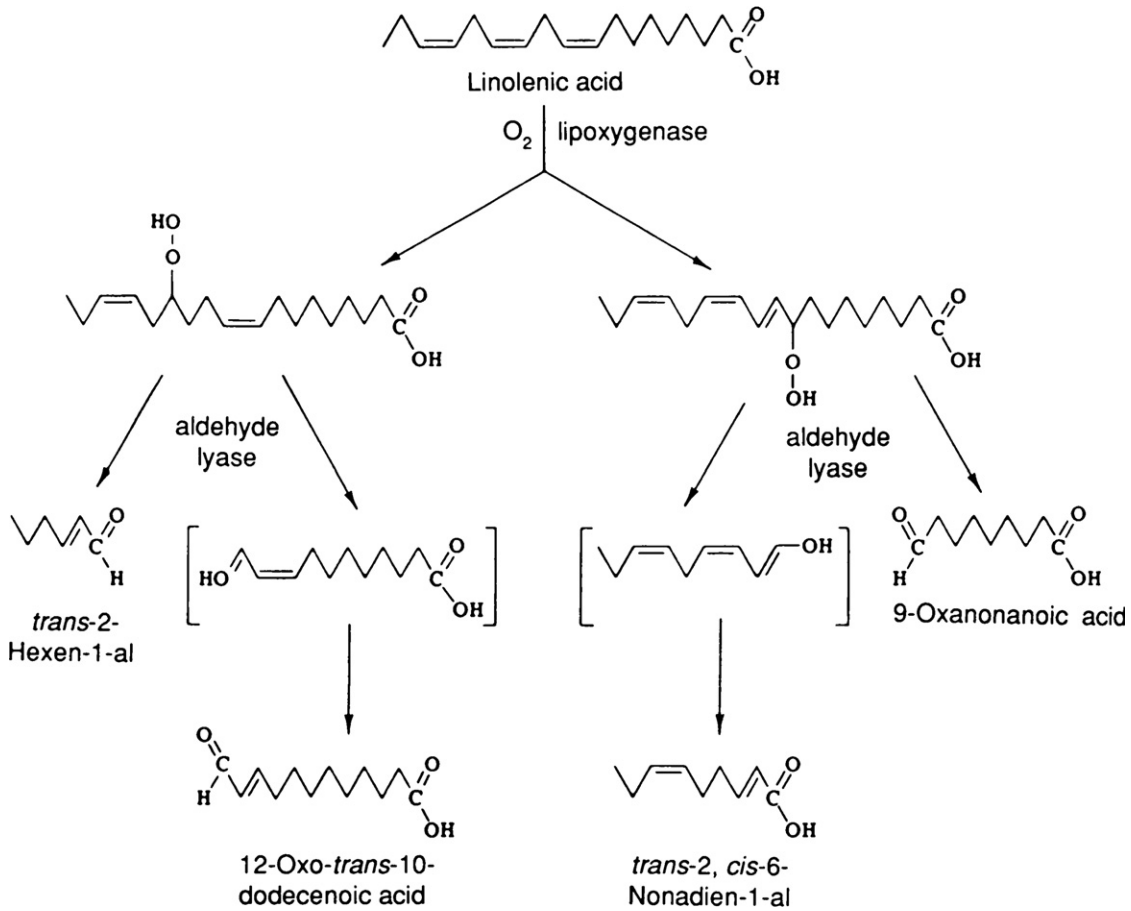


SCHEME 2.25 Proposed pathway for the formation of carbonyl fragments by the enzymatic degradation of acyl lipids in disrupted tomato fruits (Galliard and Matthew, 1977).

isolation, they overcame the problem of isomerization of *cis*-3-hexenal to *trans*-2-hexenal reported by Kazeniac and Hall (1970). Using their procedure, which involved Tenax trapping and $CaCl_2$ enzyme deactivation, they identified *cis*-3-hexenal among the major volatiles present. These researchers attributed the lack of flavor in tomatoes purchased in the supermarket to the lower levels of *cis*-3-hexenal present compared to the higher levels present in vine-ripened tomatoes. In addition, they reported that storing tomatoes in the refrigerator caused further loss of flavor in part because of lower levels of *cis*-3-hexenal. This effect of cold storage on tomato flavor was in agreement with previous work by Lammers (1981). In addition to *cis*-3-hexenal, other important flavor volatiles were β-ionone, 1-penten-3-one, hexanal, *cis*-3-hexanol, *trans*-2-hexanal, 2- and 3-methylbutanol, 2-iso-butylthiazole, and 6-methyl-5-hepten-2-one.

Trans-2-hexenal was also identified among the volatiles of 'Gros Michel' bananas by Issenberg and Wick (1963). Using a volatile enrichment technique, Tressl and Jennings (1972) confirmed the presence of *trans*-2-hexenal in the headspace of ripening bananas. Separation of volatile fractions from bananas by Palmer (1971) showed the presence of *cis*-3-hexenal, *trans*-2-hexenal, and *n*-hexanal among the aldehydes formed. Tressl and Drawert (1973) found that green banana homogenates produced *trans*-2, *cis*-6-nonadienal, *trans*-2-nonenal, and 9-oxanonanoic acid, similar to that reported in cucumbers (Fleming *et al.*, 1968). Tressl and Drawert (1973) detected hexanal, *trans*-2-hexenal, and 12-oxo-*trans*-10-dodecenoic acid when green bananas were exposed to ethylene and stored for 4 days at 15°C. Incorporation of ^{14}C-labeled linoleic and linolenic acids into these volatiles pointed to the involvement of lipoxygenase. Labeled 13- and 9-hydroperoxyoctadecadienoic acids incubated with crude banana extracts were converted to C_6–C_9 aldehydes as shown in Scheme 2.26. Aldehyde lyase, the enzyme responsible for the breakdown of the hydroperoxy derivatives, was also identified in germinating watermelon seedlings by Vick and Zimmerman (1976). This enzyme catalyzed the formation of 12-oxo-*trans*-10-dodecenoic acid and hexanal from 13-hydroperoxy-*cis*-9,*trans*-11-octadecanoic acid. This enzyme differed from hydroperoxide cleavage enzyme in tomatoes by producing *trans*-2-enals as the primary products.

Studies on apple volatiles by Flath *et al.* (1967) identified 20 compounds from 'Delicious' apple essence to be 'character impact' compounds, including hexanol, *trans*-2-hexenal, and ethyl-2-methyl butoneate. Dürr and Röthlin (1981) showed that a mixture including these and some additional components, amounting to a total of 11 components, was sufficient to compose a typical apple-like odor. Kim and Grosch (1979) partially purified



SCHEME 2.26 Reaction scheme for enzymatic splitting of linolenic acid into aldehydes and oxo acids. (Reprinted with permission from Tressl and Drawert, 1973. Copyright © by the American Chemical Society.)

lipoxygenase from apple homogenates, which produced 13-hydroperoxyoctadeca-9,11-dienoic acid from linoleic acid. The latter was converted to 2-hexenal and hexanol in a similar manner to that described previously.

B. Taste

The characteristic taste of fruits is determined by the content of sugars and organic acids. The ratio of sugar to acid is particularly useful as an index of ripeness for many fruits. Thus, studies concerning fruit quality often rely on measurements of sugars, acids, total soluble solids (°Brix), and titratable acidity (Harker *et al.*, 2002; Hoehn *et al.*, 2003). The Thiault quality index for 'Golden Delicious' apples, for example, is calculated based on total sugar content plus 10 times the acidity (Thiault, 1970). The level of sucrose and L-malic acid together with protein patterns was suggested by Gorin (1973) as parameters for assessing the quality of 'Golden Delicious' apples. Hammett *et al.* (1977) found a high correlation between the ratio of soluble solids to acid content with days from full bloom (DFFB) for 'Golden Delicious' apples. The ratio of sugars or soluble solids and acids indicates the quality of citrus and other fruits (Vangdal, 1985; Fellers, 1991; Mitchell *et al.*, 1991). Sugars and acids not only contribute to sweetness and sourness in tomatoes but are responsible for the overall flavor intensity (Kader *et al.*, 1977; Stevens *et al.*, 1979; Jones and Scott, 1983). Sweetness and hence sugars contribute to consumer liking of carrots. Studies on carrots indicated that sugars, in addition to eliciting sweetness, suppressed the bitterness exerted by 6-methoxymellein (isocumarin) (Seljasen *et al.*, 2001a, b; Höhn *et al.*, 2003; Varming *et al.*, 2004; Kreutzmann *et al.*, 2007).

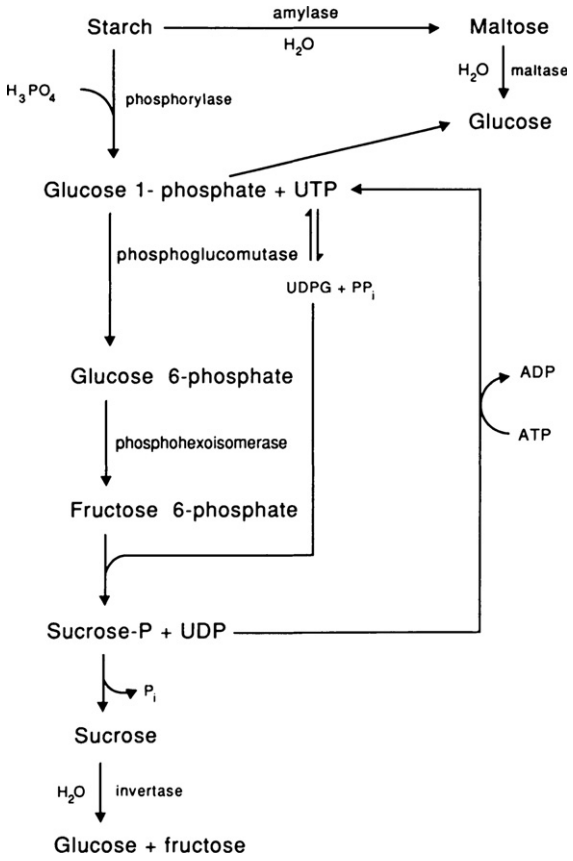
In addition to these components, the presence of tannins, phenolic compounds classified into hydrolyzable and non-hydrolyzable, affects taste (Lesschaeve and Noble, 2005). Unlike the hydrolyzable tannins, which yield gallic acid and glucose by enzymatic hydrolysis, the non-hydrolyzable tannins are resistant to enzymatic hydrolysis. The latter appears to be responsible for astringency in many under-ripe fruits. The loss of astringency in persimmon was

attributed by Matsuo and Itoo (1982) to immobilization of tannin with acetaldehyde formed during ripening. No substantial changes in the composition or amount of polyphenols in the fruit of *Rubus* sp. were detected during ripening by several researchers (Haslam *et al.*, 1982; Okuda *et al.*, 1982a, b). On the basis of studies with model systems, Ozawa *et al.* (1987) proposed that the loss of astringency during fruit ripening was due, in part, to the possible interaction between polyphenols and proteins in the fruit.

1. Starch–Sugar Conversion

Sugars and transient starch are synthesized in the growing plant by photosynthesis. This is translocated, mainly in the form of sucrose, from the chloroplasts via the phloem to the growing cells in the plant, where it is resynthesized into starch. This sucrose–starch conversion appears to involve the sequence of reactions shown in Scheme 2.27.

During the postharvest period, starch is transformed into sucrose, glucose, and fructose. This is affected by the physiological condition of the fruit and vegetables as well as storage temperature and time. Starch hydrolysis is among the more conspicuous changes accompanying ripening of many climacteric fruit. For example, a drop in starch content from 22% to 1% was reported by Palmer (1971) to accompany bananas as they passed from the preclimacteric to the climacteric phase. In other fruit such as apples and pears the progress of starch degradation is used to determine ripeness stage and harvest date (Smith *et al.*, 1979; De Jager *et al.*, 1996). However, the starch content in apples and pears is less than in bananas and hence the increase in sugars due to starch conversion is usually moderate (Murayama *et al.*, 2002; Travers *et al.*, 2002). The presence of phosphorylase and amylase enzymes was observed in the storage tissues of many fruits, although their respective roles in the ripening process remained unclear. Young *et al.* (1974) observed an increase in α-amylase activity during the ripening of bananas but were unable to confirm its involvement in starch hydrolysis. β-Amylase and phosphorylase were also found during ripening but the presence of enzyme inhibitors impeded their assay during the preclimacteric phase. Yang and Ho (1958) suggested that phosphorylase had a role in starch degradation during the climacteric. This was later confirmed



SCHEME 2.27 Sugar–starch conversion.

in gamma-irradiated 'Cavendish' bananas by Surendranathan and Nair (1973). Three phosphorylases were partially purified from ripe bananas by Singh and Sanwall (1973, 1975, 1976), each exhibiting different biochemical properties.

Starch hydrolysis was examined by Chitarra and LaJollo (1981) during the ripening of hybrid 'Marmello' bananas. This hybrid exhibited abnormal behavior as the peel, color, aroma, and texture did not undergo changes normally associated with ripening. In addition, the starch content dropped to 5% during ripening because of the possible presence of inhibitors. Raising the storage temperature from 20 to 25°C accelerated the climacteric and reduced the time required to stabilize the starch level at 5% from 24.5 to 8 days. The starch level in bananas could be further reduced to 3.3% by storing at 30°C; however, the resulting fruit was overripe and deteriorated rapidly. This contrasted with 'Dwarf Cavendish' bananas, in which complete starch degradation (97.7%) occurred during the climacteric. The lower sugar content in the 'Marmello' banana was responsible for the difference in taste compared to the corresponding 'Cavendish' variety. Phosphorylase activity in 'Marmello' bananas was reported to remain constant during the preclimacteric although it paralleled the change in starch content. This differed from 'Cavendish' fruit, in which a 50% increase in phosphorylase activity was reported prior to the climacteric and preceded any starch degradation. These results pointed to the possible involvement of phosphorylase in starch—sucrose transformations as well as sucrose synthetase (Areas and LaJollo, 1981). The exclusive involvement of α-amylase in starch degradation was seriously questioned since it occurred before any increase in α-amylase activity. The inhibition of α-amylase synthesis by cycloheximide during banana ripening did not block starch degradation, which supported a role for phosphorylase.

Phosphorylase activity has been associated with the cold storage of potato tubers at 4°C (Hyde and Morrison, 1964). This enzyme is responsible for the initial reaction in the cold-induced sweetening of potatoes (Isherwood, 1976). The overall result is a marked increase in the sugar content of the potato, resulting in the production of chips with unacceptable dark coloration (Talburt and Smith, 1975). To prevent this, potatoes are generally stored at or above 10°C, referred to as 'conditioning'. The biochemical mechanism involved in these starch/sugar transformations remains unclear, although the effect of storage temperature on a number of enzymes has been reported (Kennedy and Isherwood, 1975; Pollock and ap Rees, 1975; Isherwood, 1976; Dixon and ap Rees, 1980). Increase in sugar phosphates and sucrose was attributed by Pollock and ap Rees (1975) to the cold lability of some of the glycolytic enzymes. One of these, phosphofructokinase, was shown to be a major cause of low-temperature sweetening of potato tubers (Dixon *et al.*, 1981). The cold lability of this enzyme appeared to be due to denaturation of its oligomeric enzyme complex and its dissociation into subunits. This resulted in the inability of the enzyme to oxidize hexose phosphates, with their subsequent accumulation and conversion to sucrose.

2. Organic Acids

Fruit ripening is accompanied by changes in organic acids. These reach a maximum during the growth and development of the fruit on the tree, but decrease during storage, as well as being highly dependent on temperature. The Krebs cycle is active in the cells of higher plants in generating a variety of organic acids, including citric, malic, and succinic acids. Citric and malic acids are important constituents of most fruits, with oranges, lemons, and strawberries being high in citric acid, and apples, pears, and plums high in malic acid. During ripening, these organic acids decrease as they are used as substrates for respiration. Depletion of acids is accompanied by a decrease in starch content and an increase in sugars responsible for fruit sweetness and diminishing of acid taste (Schifferstein and Frijters, 1990; Ackermann *et al.*, 1992; Harker *et al.*, 2002). Akhavan and Wrolstadt (1980) reported that ripening 'Bartlett' pears attained a maximum sugar content of 13.5% (Figure 2.13), and a total acidity of 6 milliequivalents and flesh firmness of 6 lbs on the fourth day of ripening. Malic and citric acids accounted for the major changes in total acidity both before and after ripening (Figure 2.14). Similar evolvement of sugars and acids accompanied by an increase in pear aroma was observed in 'Conference' pears (Höhn *et al.*, 1996). These organic acids contribute to the pH in tomatoes, which is particularly important in processing (Davies and Hobson, 1981). Picha (1987) reported citric and malic acids to be the major organic acids in cherry tomato fruit. During ripening, changes in these organic acids were evident, with citric acid increasing from the immature green to mature green stage while malic acid decreased from the mature green to the table-ripe stage. Furthermore, acid and sugar levels affect the flavor quality of tomatoes and other fruits (Malundo *et al.*, 1995).

In addition to these organic acids, ascorbic acid is prominent in fruits. It is present in plant tissues, mainly in the reduced form, but can be oxidized to dehydroascorbic acid by the action of ascorbic acid oxidase (see Chapter 10).

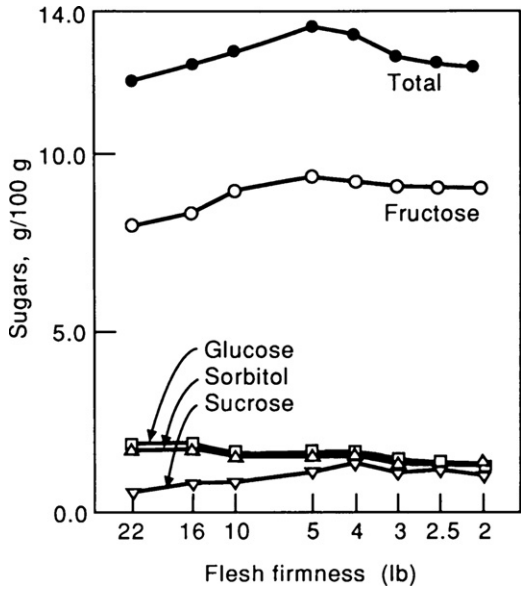


FIGURE 2.13 Changes in sugars during the ripening of Bartlett pears (Akhavan and Wrolstad, 1980). *(Copyright © by Institute of Food Technologists.)*

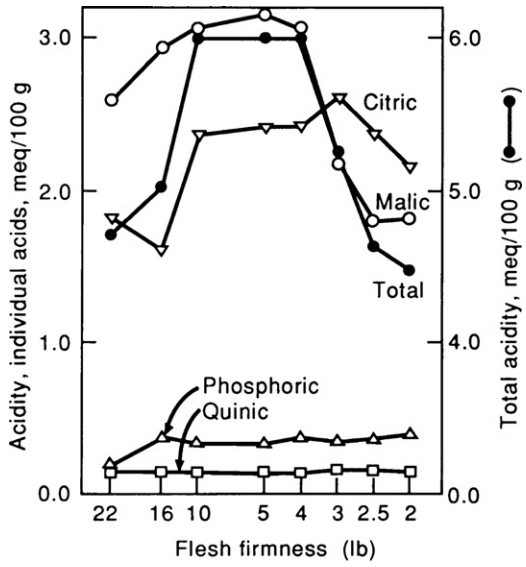


FIGURE 2.14 Changes in acids during the ripening of Bartlett pears (Akhavan and Wrolstad, 1980). *(Copyright © by Institute of Food Technologists.)*

The presence of L-quinic acid as a minor organic acid has also been reported in a number of fruits, including pears (Akhavan and Wrolstad, 1980). Other organic acids reported include oxalic and citramalic acids.

VIII. STORAGE

To ensure that an adequate year-round supply of fruits and vegetables is available for the consumer, as well as the food industry, various storage methods have been developed. The oldest of these involves cold or refrigerated storage of the perishable produce, while the others are modified atmosphere (MA) or modified atmosphere packaging (MAP) and controlled atmosphere (CA) storage. The latter technology is expensive in terms of storage facilities, equipment, and maintenance. Common to all these technologies is that fruits and vegetables remain alive and carry on processes of all living tissues, in contrast to other preservation techniques such as heat processing, dehydration, or freezing, and others which arrest respiration and other metabolic processes. Commercial interest in the development and

application of MA and CA for transportation and storage of fruits and vegetables has triggered extensive research in these fields in recent decades and has led to continuous development and refining, resulting in continuing and increasing application of these technologies worldwide.

A. Cold Storage

The oldest and most popular method for prolonging the shelf-life of perishable produce is cold storage. This is based on the fact that biological reactions, such as respiration and other metabolic processes, as well as decay, depend on temperature. The Van't Hoff rule indicates that the velocity of a reaction decreases two- to three-fold for every 10°C decrease in temperature. Thus, storage of fruits and vegetables at the lowest, but above freezing, temperatures would seem most beneficial in terms of maximal storage duration. However, some commodities show chilling injuries when exposed to low but not freezing temperatures. Most tropical or subtropical, and some crops of temperate zones, are sensitive to chilling injury. Thus, physiological and biochemical alteration may occur in sensitive species in response to low-temperature exposure and result in the development of chilling injury symptoms, such as surface pitting, discoloration, internal breakdown and flesh browning, failure to ripen, and loss of flavor, as well as decay (Ontario Ministry of Agriculture, 1998; Perez-Tello *et al.*, 2009; Schotsmans *et al.*, 2009). The critical temperature for chilling injury varies with the commodity and cultivar, and is thus genetically determined. Furthermore, the sensitivity of crops may be affected by preharvest conditions such as climatic factors and cultural practices. Officially recommended storage conditions for fruits and vegetables take this into account and, accordingly, storage temperatures recommended do not underrun critical temperatures (USDA, 2004). However, there are other factors that affect the storage life of the different commodities, including adequate relative humidity and the presence of ethylene in the storage atmosphere. Thus, for most vegetables, a high humidity must be maintained to prevent wilting or shriveling. On the other hand, a temperature range between −1°C and +3°C together with 70−75% relative humidity and good ventilation has generally been recommended for extending the storage life of onions over the winter period.

Good ventilation in a cold storeroom prevents the accumulation of ethylene or other volatile components in the store atmosphere. Ethylene increases the respiratory activity of most fruits and vegetables and hence shortens storage life. In addition, it has other beneficial as well as detrimental effects (Saltveit, 2005). Some of the detrimental effects, such as accelerated senescence, chlorophyll loss, and excessive softening, may cause unacceptable losses. Ethylene in the storage atmosphere may alter the flavor of commodities. A well-documented case describes bitterness in carrots induced by traces of ethylene (Seljasen, 2001a). Storability of commodities may be linked to the concentration of some constituents in the harvested produce; thus, Hanaoka and Ito (1957) reported that a high soluble sugar content in onion bulbs was a good indicator of their storage potential. Later researchers also found that the metabolism of carbohydrates in onions was closely linked to their storage performance (Kato, 1966; Toul and Popsilova, 1966). A more detailed study on carbohydrate metabolism in stored onions by Rutherford and Whittle (1982) showed that increased fructose resulted from the hydrolysis of the storage oligosaccharides. A low fructose content in freshly harvested onion bulbs indicated poor storage properties, with the reverse being true for onion bulbs high in fructose. Measurement of alkaline invertase in onions by Rutherford and Whittle (1984) showed that its activity reflected the level of fructose present. Monitoring fructose content and invertase activity in harvested onions provided a useful indicator for assessing their stability during cold storage.

B. Controlled Atmosphere Storage

Controlled atmosphere (CA) storage refers to storage in atmospheres different from normal air (20−21% O_2, about 0.03% CO_2, 78−79% N_2, and trace quantities of other gases) and its strict control during the entire storage time. MA and MAP rely also on storage or transportation of produce in atmospheres different from normal air, but in contrast to CA, the atmosphere is not controlled after its establishment. Thus, strict temperature control management is mandatory to effect a positive impact of MA and MAP on the quality and shelf-life of produce. Modified or controlled atmosphere conditions reduce or retard biochemical processes such as respiration, ripening, and yellowing of fruits and vegetables. The modified environment contains typically low levels of oxygen and high levels of carbon dioxide, which slow down the catabolic processes, in particular respiration, as well as aging, thereby extending the storage life of fruits and vegetables. Worth mentioning is the effect on ethylene: oxygen is required for its synthesis and both oxygen and carbon dioxide affect its biological activity. Hence, controlled or modified atmospheres slow down the synthesis and activity of ethylene during storage.

In the 1960s it became evident that maintaining as low levels of oxygen as tolerated by produce was advantageous. This was reflected in the terminology used for different storage regimes, such as LO (low-oxygen) storage or ULO (ultra-low-oxygen) storage (Hoehn *et al.*, 2009). In general, the lowest possible level of oxygen corresponds to the anaerobic compensation point (ACP) (Figure 2.15). The ACP is the oxygen concentration at which carbon dioxide production is at a minimum (Boersig *et al.*, 1988).

The overall benefit of CA storage is that produce can retain its freshness and eating quality for much longer periods. Not all fruits and vegetables, however, can be stored under these conditions. Considerable research has been conducted on a wide range of produce, but apples remain worldwide the major fruit handled commercially under CA storage conditions. Other commodities stored or shipped commercially under controlled atmospheres include pears, kiwis, berries, cabbage, onions, lettuce, avocados, asparagus, and bananas. One of the major problems associated with CA storage is that different conditions are required for the storage of different cultivars. For example, apples have been extensively studied, with lists of recommended conditions for specific cultivars available from different regions of the USA, England, and Europe (Stoll, 1970, 1973; Blanpied, 1977; Fidler *et al.*, 1977; Porritt, 1977; Kader, 2003; Kupferman, 2003; Saltveit, 2003b; Brecht, 2006; Erkan and Wang, 2006). This makes CA storage very expensive as conditions have to be tailor-made for the different cultivars of the same crop as well as for the different fruits and vegetables.

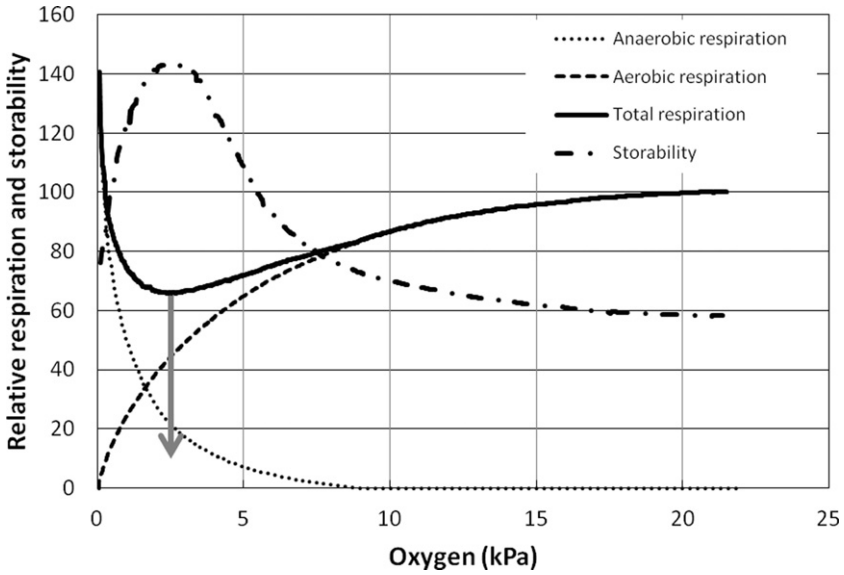


FIGURE 2.15 Effect of oxygen concentration on respiration and aerobic compensation point (ACP). The ACP is the O_2 concentration at which respiration (CO_2 production) is at a minimum. *(From Gasser et al., 2008. With permission.)*

C. New Developments in Postharvest Storage

The establishment of commercial applications of CA storage technology was based on research on the effects of oxygen, carbon dioxide, and ethylene (C_2H_2) on respiration and ripening in pome fruits and berries by Kidd and West (1927, 1930b, 1937, 1950). Since then, this technology has been continuously developed and refined (Dilley, 2006). In the new millennium two new developments were implemented. The first was the application of 1-methylcyclopropene (1-MCP) as a postharvest chemical (Sisler and Blankenship, 1996). 1-MCP is a low-boiling compound. For its commercial use it is complexed with α-cyclodextrin to produce a water-soluble powder (Daly and Kourelis, 2000). For application, the soluble powder is mixed with water, thereby releasing 1-MCP as a gas and then dispersing it around the produce. The application rates recommended are in the range of 0.2−1 ppm (Kostansek and Pereira, 2003). Treatment durations of 12−24 hours are generally sufficient to achieve a full response. Since 2002 1-MCP has been approved for postharvest application of apples in many countries (Prange and DeLong, 2003). In some countries application for bananas has been approved, while for other commodities governmental approval is pending. 1-MCP binds to the ethylene receptors and inhibits the action of both internal and external ethylene, resulting in a considerable retarding of ripening processes. However, individual commodities respond differently to 1-MCP (Watkins, 2002; Blankenship and Dole, 2003) and more research remains to be done on 1-MCP effects on both postharvest and non-postharvest processes occurring in plants.

A second new development in postharvest techniques encompasses dynamic or adaptive control of controlled atmosphere storage (DCA). Recommendations for CA and MA of fruits and vegetables are usually based on numerous empirical storage trials to find the optimal CA conditions. The conditions yielding the best storage results are normally recommended and maintained from the beginning to the end of storage (Prange *et al.*, 2005; Gasser *et al.*, 2008). Since stored fruits and vegetables are living tissues, it is reasonable to expect their metabolism to be dynamic in nature during storage. Thus, static CA conditions are not likely to be optimal and may even be incorrect at some point during the storage period. Saltveit (2003b) pointed out this aspect and advocated the need for dynamic CA. A promising technique to dynamically control CA appears to be chlorophyll fluorescence measurements (Prange *et al.*, 2003, 2005; Gasser *et al.*, 2005). Chlorophyll fluorescence is affected by low oxygen and high carbon dioxide (DeEll *et al.*, 1995, 1998) and research indicates that the technique detects the lowest level of oxygen that is tolerated by stored fruits and vegetables. This oxygen concentration would correspond to the ACP (Gasser *et al.*, 2005, 2007). Based on the principle of chlorophyll fluorescence, the Harvest Watch™ system has been developed and subsequently patented (Prange *et al.*, 2007). The first commercial applications of DCA for apples were undertaken in 2004 in Italy and the USA and, since then, its application for storage of apples has increased steadily worldwide. Storing apples just above oxygen threshold concentrations (ACP) resulted in improved quality retention and it has been reported that this technology maintains apple quality after storage equal to, or better than, 1-MCP (Zanella *et al.*, 2005). Adaptive control of CA conditions entails monitoring the response of horticultural produce to changes in storage parameters or stressful conditions. Another indicator represents the fermentation threshold. It can be monitored and deduced from increases of acetaldehyde or ethanol in the storage atmosphere. Devices to monitor ethanol have been developed and were applied in the CA storage of 'Elstar' apples in the Netherlands (Veltman *et al.*, 2003c). Other techniques may be incorporated into CA systems in the future, for example, control of storage rots by natural antimicrobial compounds delivered as gas at critical periods during storage, or the addition of novel gases (NO, N_2O) to increase the benefits of current CA (Hoehn *et al.*, 2009).

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