Chapter 4

Milk

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Chapter Outline

I. INTRODUCTION

Milk and dairy products are important sources of animal protein, vitamins, minerals, and essential fatty acids for infants and young adults (Walstra *et al.*, 2006). The major source of milk is obtained from Western breeds of dairy cattle (*Bos taurus*), although milk is available from other species of mammal in some countries. According to the Pasteurized Milk Ordinance of the United States Public Health Service (2011), milk is defined as the normal lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, containing not less than 8.25% milk solids-not-fat (SNF) and not less than 3.25% of milk fat. The exclusion of colostrum, a fluid secreted immediately following parturition, is primarily for esthetic reasons. Over 70 billion gallons of milk are produced annually in the USA, with more than 30 billion gallons consumed as liquid milk (Goff and Griffiths, 2006).

Since the publication of the second edition there have been major advances in our understanding of the mechanism of milk secretion. These include the intracellular assembly of milk droplets and the nature and formation of the milk fat globule membrane (MFGM), as well as the regulation of the mammary synthesis of milk fat (Bauman *et al.*, 2006). Several comprehensive advanced dairy chemistry texts have also been published (Fox and McSweeney, 2003, 2006, 2009; Walstra *et al.*, 2006). This chapter will discuss the dynamic biochemical systems involved in the biosynthesis of milk components.

II. COMPOSITION OF MILK

The major constituents of milk are water (86−88%), milk fat (3−6%), protein (3−4%), lactose (5%), and minerals (ash) (0.7%), with total solids of 11−14%. The composition of milk is affected by a variety of factors, including breed, stage of lactation, nutritional and health status of the cow, season (which can be both temperature and stage of lactation effects, if calving is not even throughout the year), and genetic factors (Fox and McSweeney, 1998). Table 4.1 illustrates the influence of the four main breeds in North America on yield and milk composition and also

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TABLE 4.1 Changing Yield and Composition of Milk Over the Past Two Decades in Canada, as a Function of Breed

	All Breeds			Holstein			Jersey			Guernsey			Brown Swiss		
Year	Yield (kg)[a]	Fat (%)	Protein (%)	Yield (kg)	Fat (%)	Protein (%)	Yield (kg)	Fat (%)	Protein (%)	Yield (kg)	Fat (%)	Protein (%)	Yield (kg)	Fat (%)	Protein (%)
2009[b]	9592	3.77	3.22	9793	3.76	3.19	6371	4.87	3.81	6812	4.56	3.43	8128	4.05	3.48
2008	9642	3.78	3.23	9836	3.74	3.20	6435	4.84	3.81	6820	4.51	3.45	8366	4.04	3.48
2007	9538	3.77	3.22	9733	3.72	3.19	6412	4.82	3.78	6673	4.51	3.46	8159	4.06	3.48
2006	9481	3.75	3.21	9677	3.71	3.18	6331	4.83	3.77	6540	4.55	3.43	8064	4.06	3.46
2005	9422	3.76	3.21	9624	3.71	3.19	6279	4.85	3.77	6398	4.50	3.43	7792	4.12	3.48
2004	9458	3.72	3.22	9658	3.67	3.19	6291	4.85	3.77	6435	4.54	3.45	8048	4.07	3.47
2003	9519	3.73	3.23	9721	3.68	3.21	6344	4.87	3.81	6570	4.49	3.49	8038	4.04	3.49
2002	9511	3.72	3.25	9717	3.67	3.22	6407	4.86	3.84	6347	4.44	3.51	8215	4.03	3.50
2001	9242	3.72	3.24	9440	3.68	3.22	6186	4.87	3.83	6015	4.45	3.51	8020	4.02	3.50
2000	9152	3.70	3.23	9350	3.67	3.21	6203	4.90	3.83	5949	4.45	3.48	7920	3.96	3.48
1999	8960	3.69	3.24	9162	3.66	3.22	6072	4.89	3.85	5939	4.43	3.54	7585	4.03	3.54
1998	8738	3.70	3.24	8946	3.65	3.22	6002	4.88	3.84	5991	4.48	3.55	7105	4.04	3.50
1997	8427	3.72	3.24	8697	3.68	3.22	5753	4.90	3.86	5919	4.53	3.55	6818	4.05	3.52
1996	8424	3.76	3.25	8633	3.72	3.23	5720	4.93	3.86	5984	4.56	3.54	6910	4.08	3.53
1995	8251	3.74	3.24	8461	3.70	3.21	5620	4.89	3.86	5936	4.57	3.54	6709	4.05	3.49
1994	8103	3.74	3.26	8309	3.69	3.21	5501	4.94	3.94	5867	4.76	3.56	6718	4.06	3.51
1993	7988	3.75	3.25	8193	3.71	3.21	5408	4.94	3.88	5826	4.58	3.57	6639	4.07	3.52
1992	7807	3.73	3.24	8028	3.67	3.21	5244	4.92	3.91	5755	4.55	3.58	6483	4.03	3.48
1991	7523	3.70	3.21	7717	3.66	3.22	4992	4.90	3.92	5554	4.52	3.60	6176	4.01	3.54

[a] *Based on a 305 day lactation period.*
[b] *In 2009, Holsteins accounted for 92.8% of the population, Jerseys 3.2%, Guernsey < 1%, and Brown Swiss < 1%.*
Source: Canadian Dairy Information Centre, www.dairyinfo.gc.ca (2010).

shows the changes in yield and composition that have occurred over two decades owing to improved genetics and nutrition. Holsteins produce by far the majority of milk; although they are lower in fat and protein on a percentage weight basis, the yield is much higher, so they produce a higher total amount of fat and protein. Seasonal changes in fat, protein, and lactose and other solids can be seen in Table 4.2 from all milk produced in the Province of Ontario in 2006. Since calving is evenly distributed, these changes are due to temperature and feeding regimes. In countries where calving is more heavily concentrated in certain periods, such as spring, milk composition across the seasons can also be affected by stage of lactation.

Extensive studies on milk composition in North America were undertaken because of considerable interest in the percentage of SNF or protein in determining milk prices. This measurement was facilitated through the development of rapid, automated methods based on infrared analysis. Wilcox *et al.* (1971) completed a large study on milk composition in five breeds and reported that the effect of age on fat, SNF, protein, and total solids (TS) was as important as its effect on milk yield. This was followed by Norman *et al.* (1978), who examined the influence of age and month of calving on milk fat, SNF, and protein yields over a period of nine years. This was the largest study ever conducted and involved 106,411 cows from 2215 herds in 41 states. These researchers found that the levels of milk components were lower than those reported by Wilcox *et al.* (1971) but reflected more closely the average US yields for milk and fat. The average percentages for fat and SNF reported by Norman *et al.* (1978) were 3.8% and 8.6%, respectively. The effect of breed on milk composition was consistent with previous studies. A later study by Sommerfeldt and Baer (1986) monitored the variation in milk components from 1705 herds from eastern South

TABLE 4.2 Average Monthly Milk Composition (kg/hl) from the Province of Ontario, Canada

Month	Milk Fat	Milk Protein	Lactose and Other Solids
January	3.99	3.34	5.71
February	4.00	3.32	5.72
March	4.01	3.34	5.73
April	3.97	3.30	5.74
May	3.91	3.27	5.75
June	3.85	3.24	5.73
July	3.77	3.20	5.72
August	3.78	3.25	5.70
September	3.92	3.33	5.70
October	4.04	3.37	5.70
November	4.03	3.39	5.70
December	4.01	3.33	5.70

Data from University of Guelph Laboratory Services, http://www.guelphlabservices.com (2006).

Dakota, Minnesota, and northern Iowa. Milk was collected biweekly over a one-year period to evaluate the advantages and disadvantages of milk payment based on SNF. The greatest variability was observed for fat (8.4% coefficient of variation), followed by protein, TS, and SNF, with coefficients of variation of 6.3%, 4.1%, and 3.4%, respectively. Sommerfeldt and Baer (1986) did not find any consistent relationship between SNF and protein or fat in the milk examined and recommended that testing for SNF should also include the fat content and producer grade. The introduction of a milk component pricing plan was considered to be beneficial to both the producer and processor as milk high in protein would be particularly attractive to cheese manufacturers as this would lead to higher yields.

During the past decade, Stoop *et al.* (2006, 2008) showed the potential for selective breeding to alter milk composition as genetic effects showed a high heritability for lipid (0.52), protein (0.60), and lactose (0.64) contents, and moderate heritability for milk (0.44), lipid (0.37), and protein (0.34) yields. Using elite dairy cows (Swedish Red and Swedish Holstein breeds), classified by their high genetic merit, Glantz *et al.* (2009) reported higher yields for components of protein, lipids, carbohydrate profiles, and minerals. However, the content of milk components such as lipids and whey was found to decrease on average. Milk from these cows exhibited good gelation characteristics but was more susceptible to oxidation owing to a lower antioxidant capacity. This study showed that milk composition and processing characteristics could be used to adjust breeding practices for optimizing milk quality and stability.

A study in the Netherlands by Heck *et al.* (2009) examined the effect of seasonal variation on the detailed composition of milk from February 2005 to February 2006. Milk protein ranged from a low of 3.21 g/100 g in June to a high of 3.38 g/100 g in December, while milk fat increased from a low of 4.10 g/100 g in June to a high of 4.57 g/100 g in January. The largest seasonal changes were observed for fatty acid composition in which *trans* fatty acids, including conjugated linoleic acid, increased up to two-fold. In contrast, however, milk lactose remained unchanged throughout the seasons.

While environmental and physiological factors have been shown to affect milk fat, it is nutrition that appears to be the dominant factor (Bauman *et al.*, 2006). The most dramatic effect of nutrition is the low-fat milk syndrome, often referred to as milk fat depression (MFD). Reduction in milk fat was associated with diets containing fish and plant oil supplements as well as those high in concentrates and low in fiber.

III. MILK CONSTITUENTS

The overall composition of cow's milk provides useful information to the farmer and processor in determining quality and market value. This section will discuss the chemistry and biochemistry of major components in milk.

A. Lipids

The lipid component of cow's milk (3−5%) is secreted by the lactating cow as small fat globules ranging in size from < 1 to 15 μm in diameter. Each fat globule is surrounded by a special interfacial layer, or milk fat globule membrane (MFGM), composed of a lipid bilayer and protein (Spitsberg, 2005; Singh, 2006). This layer ensures that each lipid droplet is dispersed in the aqueous milk serum and is unable to aggregate with others. Kanno (1990) reported that the proteins and lipids in MFGM are present in a 1:1 weight ratio. Earlier work by Wooding and Kemp (1975) suggested that MFGM lipids contain a large amount of high-melting triacylglycerols, as indicated in an MFGM membrane model proposed by McPherson and Kitchen (1983). However, Walstra (1974, 1985) reported that this was an artifact caused by contamination of MFGM fragments with triacylglycerol crystals during the isolation procedure. MFGM lipids are now recognized to be predominantly polar, with a very small amount of neutral lipids including triacylglycerols, diacylglycerols, monoacylglycerols, and cholesterol and its esters. The overall gross composition of MFGM is shown in Table 4.3. A review of the literature shows that values vary considerably owing to differences in the isolation, purification, and analytical procedures used (Dewettinck *et al.*, 2008). The MFGM layer also contains at least 50 polypeptides ranging in size from 10 to 300 kDa (Spitsberg and Gorwitt, 1998; Mather, 2000) as well as trace elements.

The structure of MFGM, from the lipid core outwards, was shown schematically by Dewettinck *et al.* (2008) and is reproduced in Figure 4.1. The inner core consists of a monolayer of polar lipids and proteins surrounding the intracellular fat droplet with an electron-dense proteinaceous coat on the inner face of the bilayer membrane with a true bilayer of polar lipids and proteins. The phospholipid bilayer appears to serve as the backbone of the membrane and exists in the fluid state. Choline-containing phospholipids together with glycolipids, cerebrosides, and gangliosides are found predominantly on the outside of the membrane, with phosphatidyl ethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) concentrated on the inner surface of the membrane. Partially embedded or loosely attached to the bilayer are peripheral membrane proteins. Of these, adipophilin (ADPH) is located in the inner polar layer, while xanthine dehydrogenase/oxidase (XDH/XO) is found between the two layers. Other major MFGM proteins, mucin 1 (MUCI), butyrophilin (BTN), cluster of differentiation 36 (CD36), and periodic acid/Schiff III (PAS III), are shown in the outer layer, with periodic acid/Schiff 6/7 (PAS 6/7) and proteose peptone 3 (PP3) loosely attached to the outside of the membrane. For a more detailed discussion of MFGM structure, the following papers are recommended: Dewettinck *et al.* (2008), Evers *et al.* (2008), Reinhardt and Lippolis (2008), Sanchez-Juanes *et al.* (2009), and Vanderghem *et al.* (2008).

The amphiphilic nature of the polar lipids (phospholipids and sphingolipids) in the MFGM layer has an important influence on the stability of the fat phase in milk and the changes that occur during the processing of milk and cream. McPherson and Kitchen (1983) summarized the factors affecting the composition of the MFGM layer as shown in Scheme 4.1. The effect of some of these compositional changes on the properties of the MFGM layer remains

TABLE 4.3 Estimated Average Composition of the Milk Fat Globule Membrane

Component	mg/100 g Fat Globules	g/100 g MFGM Dry Matter
Proteins	1800	70
Phospholipids	650	25
Cerebrosides	80	1
Cholesterol	40	2
Monoglycerides	+[a]	?
Water	+	−
Carotenoids + vitamin A	0.04	0.0
Iron	0.3	0.0
Copper	0.01	0.0
Total	> 2570	100

MFGM: milk fat globule membrane.
[a]*Present, but quantity unknown.*
From Dewettinck et al. *(2008).*

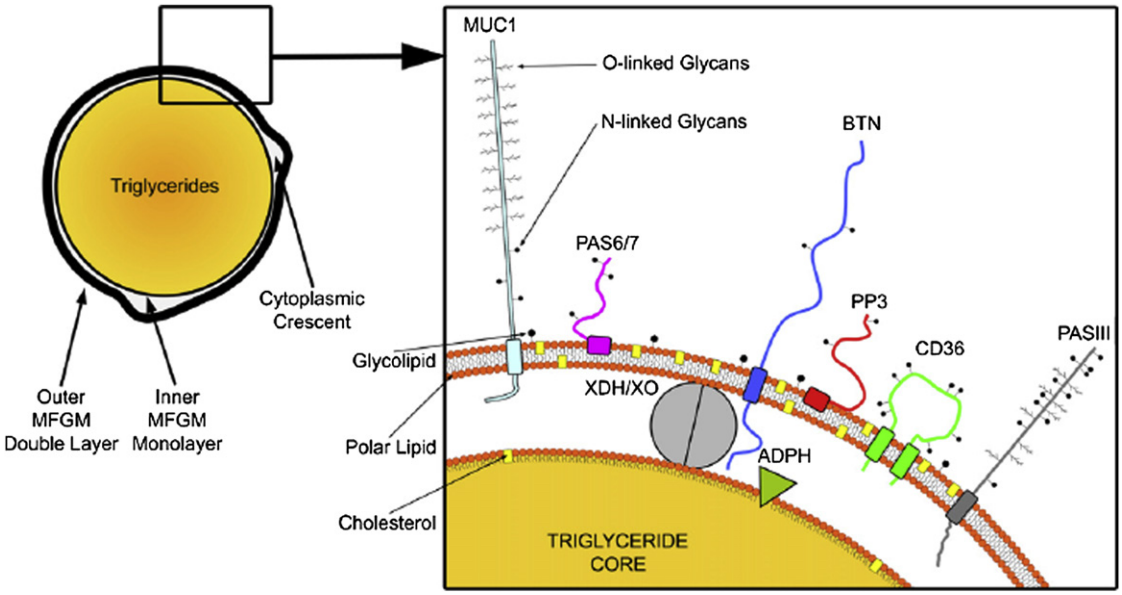
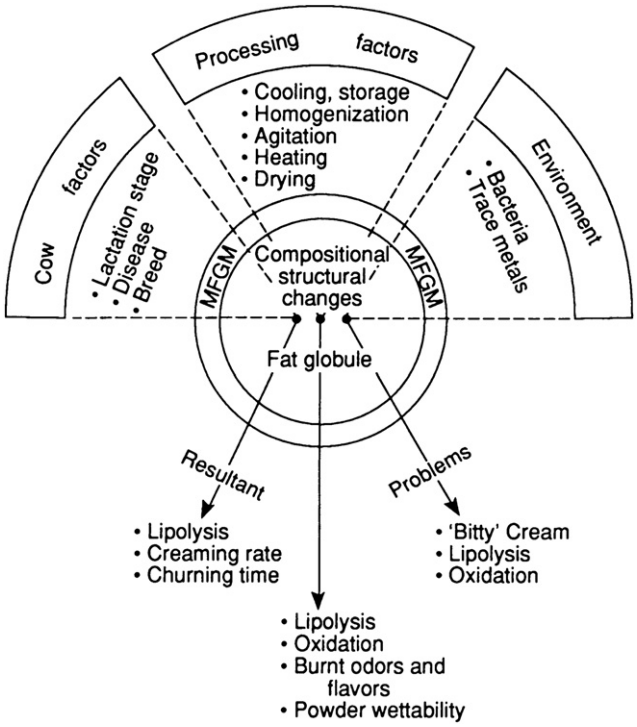


FIGURE 4.1 Structure of the fat globule with detailed arrangements of the main milk fat globule membrane (MFGM) proteins. The drawing is highly schematic and sizes are not proportional. A double layer of polar lipids is placed on the inner monolayer of polar lipids. Membrane-specific proteins are distributed along the membrane. Adipophilin (ADPH) is located in the inner polar lipid layer. Xanthine dehydrogenase/oxidase (XDH/XO) is located between the two layers. Mucin 1 (MUC1), butyrophilin (BTN), cluster of differentiation 36 (CD36), and periodic acid/Schiff III (PAS III) are located in the outer layer. Periodic acid/Schiff 6/7 (PAS 6/7) and proteose peptone 3 (PP3) are only loosely attached to the outside of the MFGM. The choline-containing phospholipids, PC and SM, and the glycolipids, cerebrosides and gangliosides, are largely located on the outside of the membrane, while phosphatidyl ethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are mainly concentrated on the inner surface of the membrane *(Deeth, 1997; Mather and Keenan, 1998; Danthine* et al.*, 2000; Mather, 2000; Harrison, 2002; Rasmussen* et al.*, 2002; Evers, 2004; Dewettinck* et al.*, 2008).*



SCHEME 4.1 Factors affecting the milk fat globule membrane. *(McPherson and Kitchen, 1983)*

unclear. The outer surfaces of the MFGM are quite labile and can be removed by simple washing procedures and by temperature manipulation. Such losses of the outer surface have an impact on the processing and storage of milk.

Triacylglycerols account for 97−98% of the total milk fat. The fatty acid composition of the triacylglycerols is influenced by breed, management practices, and season, with saturated fats being lower in summer than in winter (Table 4.4). Milk fats are not markedly affected by changes in dietary lipids since some lipids are metabolized by the microbial activity in the cow's rumen (Garton, 1964). Nevertheless, modification of milk fat can be achieved by feeding the cow a triacylglycerol emulsion protected by a cross-linked protein membrane, to protect the fat from microbial metabolism in the rumen. This technique allows the fatty acid composition of the milk fat to be manipulated and the degree of unsaturation of fats to be modified. The fatty acid composition of bovine milk lipids is more varied than that of any other natural product, with 437 components listed by Patton and Jensen (1975). Of these,

TABLE 4.4 Fatty Acid Composition (% w/w) of Total Fatty Acids in Swedish Dairy Milk in 2001, given as Weighted Mean with Standard Deviation (SD); the Lowest and Highest Values Observed and *p*-Values for Geographical and Seasonal Variation are also given (Mansson, 2008)

Fatty Acid	Weighted Mean 2001	SD	Lowest Value Observed	Highest Value Observed	Seasonal Variation
4:0	4.4	0.1	4.0	5.1	n.s.
6:0	2.4	0.1	2.1	2.9	n.s.
8:0	1.4	0.1	1.2	1.9	n.s.
10:0	2.7	0.2	2.4	3.5	*
12:0	3.3	0.2	3.0	4.1	**
14:0	10.9	0.5	10.0	12.1	***
15:0	0.9	0.0	0.8	1.1	n.s.
16:0	30.6	0.9	28.7	34.1	**
17:0	0.4	0.0	0.4	0.5	**
18:0	12.2	0.4	10.3	13.3	n.s.
20:0	0.2	0.0	0.2	0.2	n.s.
Saturated fatty acids, total	69.4	1.7	67.1	74.4	***
10:1	0.3	0.0	0.2	0.4	n.s.
14:1	0.8	0.4	0.4	1.3	**
16:1	1.0	0.0	0.9	1.8	n.s.
17:1	0.1	0.0	< 0.1	0.3	n.s.
18:1	22.8	1.0	19.7	24.7	***
Monounsaturated fatty acids, *cis*, total	25.0	1.0	22.2	26.7	**
18:2	1.6	0.1	1.4	1.8	n.s.
18:3	0.7	0.0	0.6	0.9	**
Polyunsaturated fatty acids, *cis*, total	2.3	0.1	2.0	2.5	n.s.
16:1t	0.4	0.1	0.3	0.4	***
18:1t	2.1	0.7	2.0	3.3	***
18:2t	0.2	0.0	0.1	0.5	n.s.
Trans fatty acids, total	2.7	0.7	0.6	3.9	***
CLA	0.4	0.1	0.3	0.5	***

n.s.: Not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

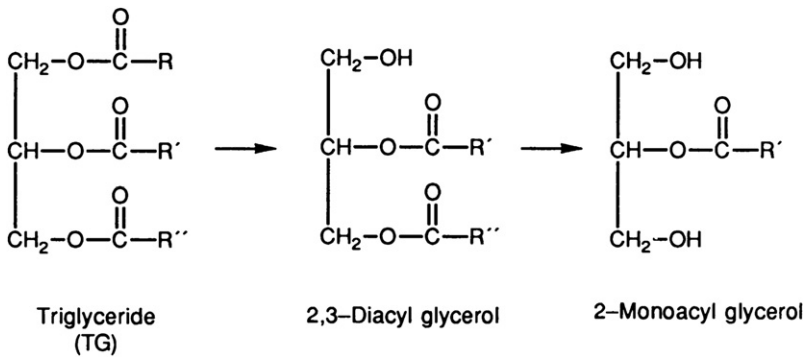
saturated fatty acids account for 70%, monounsaturates 25%, *cis*-polyunsaturates 2.3%, and *trans* fatty acids 2.7%. Long-chain fatty acids (C14−C23) and short-chain fatty acids (C4−C6) account for 85% and 15% of the total fatty acids in the triacylglycerols of milk fat, respectively (Table 4.4).

1. Biosynthesis of Milk Fat

The synthesis of milk triacylglycerols relies on blood lipids and *de novo* synthesis in the mammary epithelial cells. The majority of C16−C18 fatty acids in bovine milk triglycerides are derived from dietary fat taken up from the blood, while the C4−C14 fatty acids appear to be synthesized *de novo* in the mammary gland. The dietary fatty acids are transported from the intestine to the mammary glands and other tissues by chylomicrons (Zinder *et al.*, 1974). These chylomicrons are formed in the intestinal epithelial cells from lipids absorbed in the intestine and then transported by the lymph in the intestinal lymphatic vessels to the thoracic duct via the bloodstream, to be taken up by various tissues of the body. Chylomicrons are spherical particles consisting of a triacylglycerol core with traces of cholesteryl esters. They are enclosed by a monolayer of surface film 25−30 Å in width composed primarily of phospholipids and diglycerides with small amounts of cholesterol, protein, monoglycerides, and fatty acids (Scow *et al.*, 1976). Evidence supports the hypothesis that triacylglycerols are taken up by the mammary gland from the chylomicrons, when present, and as low-density lipoprotein (LDL; $d < 1.05$ g/ml) (Moore and Christie, 1979). Mazur and Rayssiguier (1988) reported that dairy cows have characteristically low levels of triacylglycerols and very-low-density lipoprotein (VLDL; $d < 1.01$ g/ml). Most of the circulating lipids are related to high-density lipoprotein (HDL; $d = 1.06$-1.21 g/ml), which carries only small amounts of triacylglycerols. In other words, the triacylglycerols circulating in the blood as lipoprotein ($d > 1.05$) do not donate their fatty acids to the mammary gland for milk fat synthesis. Data presented by Brumby and Welch (1970) and Stead and Welch (1975) demonstrated quite clearly that serum lipoproteins from lactating cows contained negligible amounts of triacylglycerols. In spite of all these reports, Bickerstaffe (1971) still claimed that triacylglycerols were present in all lipoprotein fractions in the blood of lactating cows and were taken up by the mammary gland.

Under normal conditions, when a standard dietary regimen is fed to the cow, the main fatty acids in the blood triacylglycerols are C16:0, C18:0, and C18:1, which are taken up by the mammary gland. Modification of the diet, by feeding canola seed protected with formaldehyde to escape biohydrogenation in the rumen, resulted in an increase in C18:1, C18:2, and C18:3 content in the plasma of Holstein cows (Delbecchi *et al.*, 2001). This increase was reflected by a corresponding increase in the assimilation of C18:1, C18:2, and C18:3 by the mammary gland. It is clear, however, that the fatty acid composition in the plasma is not the sole determinant of fatty acids in the plasma triacylglycerols. Annison *et al.* (1967) and West *et al.* (1972) both observed that the total triacylglycerol in the arterial plasma was much higher in C18:0 and lower in C18:1. This selective assimilation was confirmed by Moore *et al.* (1969), who concluded that the mammary glands selectively assimilated a plasma triacylglycerol with low levels of C18:2 in the presence of high C18:2 plasma triacylglycerols for the synthesis of milk fat.

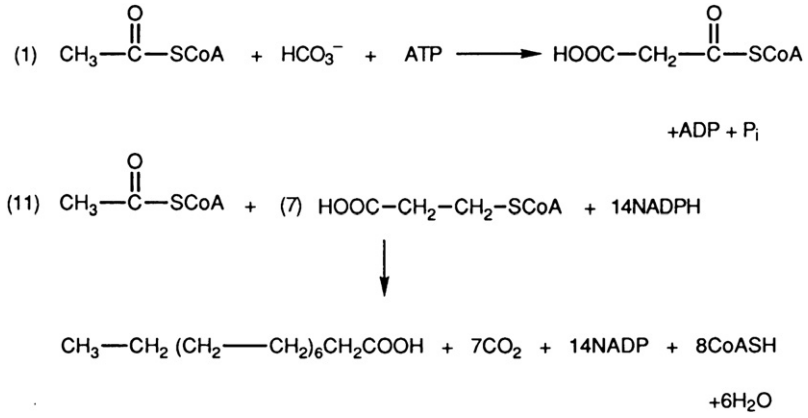
The distribution of triacylglycerol fatty acids among the different lipoprotein fractions was examined because the triacylglycerol in the chylomicrons and VLDL hydrolyzed during uptake into tissues by lipoprotein lipase. Goldberg *et al.* (2009) reviewed the regulation of fatty acid uptake by lipoprotein lipase. Lipoprotein lipase purified from cow's milk was shown by a number of researchers (Morley and Kuksis, 1972; Morley *et al.*, 1975; Paltauf *et al.*, 1974; Paltauf and Wagner, 1976) to have a high specificity for the acyl ester bond in position 1 of the triacylglycerol, hydrolyzing it to 2-monodactyl glycerol according to the following sequence:



Studies on the uptake of blood triacylglycerol by the mammary gland and the role of lipoprotein lipase have been conducted primarily with non-ruminant animals. Nevertheless, the overall results indicate that chylomicrons and VLDL triacylglycerols of mammary tissue are the substrates and apoprotein and phospholipid cofactors for optimum lipoprotein lipase activity. The enzymatic hydrolysis of triacylglycerol occurs only in the chylomicron attached to the endothelium, possibly by hydrolysis of the acyl ester bond at the 1 position. Further hydrolysis of some of the remaining diglycerides at the lumenal surface may occur, and they are then taken up into the microvesicles and transported across the endothelial cells. The second acyl ester bond of the diglyceride is hydrolyzed as the microvesicle crosses the endothelium, where some monoglyceride is also hydrolyzed to free glycerol. Once the diglycerides enter the alveolar cells of the mammary gland they are resynthesized into milk triacylglycerol. Under normal dietary conditions, a fraction of plasma unesterified fatty acids equal to that released into the bloodstream by hydrolysis of chylomicron triacylglycerol is taken up for the synthesis of milk fat (Moore and Christie, 1979).

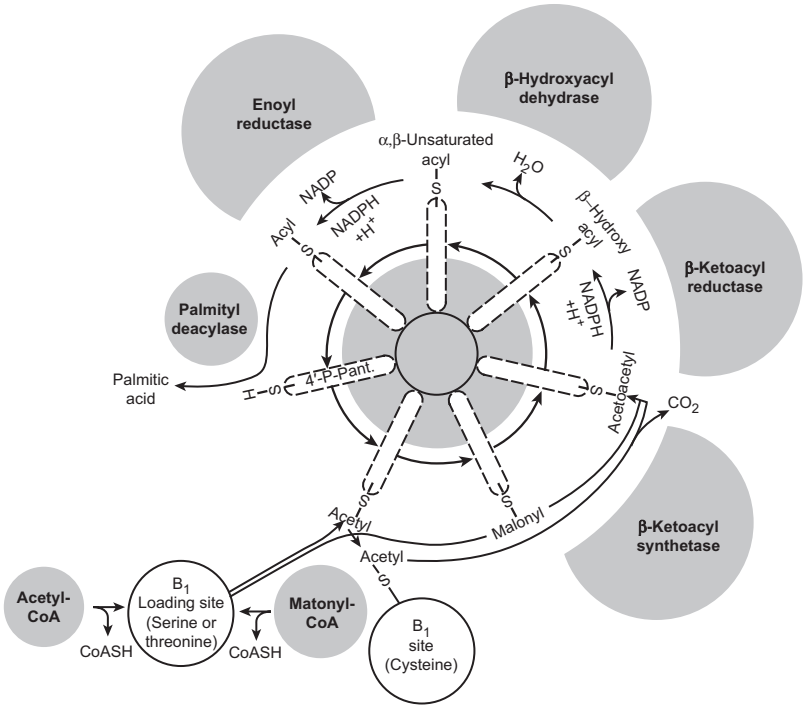
2. Fatty Acid Synthesis in the Mammary Gland

The synthesis of fatty acids in the mammary gland involves the stepwise condensation of C2 units by reversal of β-oxidation (Hele, 1954):



Reaction (1) is catalyzed by acetyl-coenzyme A (acetyl-CoA) carboxylase forming malonyl-CoA, while the second reaction (11) is mediated by a group of enzymes known collectively as the fatty acid synthetase reaction sequence. Current evidence still indicates that acetate and β-hydroxybutyrate are the two main sources for the *de novo* synthesis of fatty acids in lactating ruminants. The fatty acid synthetase sequence consists of seven enzymes integrated into a multienzyme complex, which copurifies as a single protein (Smith, 1976). In animals, fatty acid synthetase requires NADPH and produces C16:0 as the main free fatty acid. This is illustrated in Scheme 4.2, in which each cycle is initiated by the transfer of a saturated acyl group from 4-phosphopantatheine thiol to the cysteine site (B2) with the simultaneous transfer of a malonyl group from the loading site (B1) to the 4-phosphopantatheine thiol. Subsequent reactions include condensation, ketoreduction, dehydration, and enoyl reduction, with the end product being a saturated acyl moiety. To synthesize palmityl this cycle has to be completed seven times. The termination of the acyl chain occurs through the action of a deacylase or thioesterase enzyme, which hydrolyzes the acyl-4-phosphopantatheine thioester bond.

Ruminant milk fat contains substantial amounts of short- and medium-chain fatty acids, which are synthesized in the mammary gland. The synthesis of fatty acids from [^{14}C]acetate by mammary tissue obtained by biopsy from cows before and after parturition was examined with tissue slices (Mellenberger *et al.*, 1973) and homogenates (Kinsella, 1975). These researchers noted that the synthesis of short- and medium-chain fatty acids varied with the physiological state of the mammary glands. Only trace amounts of C4:0 to C10:0 fatty acids were synthesized in mammary tissue 18 days before parturition, with C16:0, C14:0, C18:0, and C12:0 accounting for 60%, 30%, 5%, and 4% of the total fatty acids, respectively. This was in sharp contrast to 7 days before parturition, when C4:0–C10:0 fatty acids accounted for 40% of the total fatty acids while C16:0 represented only 30% of the total. A 30-fold increase in the rate of fatty acid synthesis was observed in both mammary slices and homogenates, which began 18 days before parturition and continued until 20 days after parturition. Based on these studies, it is clear that some hormonal control is involved in the synthesis of specific patterns of fatty acid by the mammary glands.



SCHEME 4.2 A mechanism of fatty acid synthesis by the mammalian prototype 1 system according to J. Porter. Note the flow of acetyl-CoA and malonyl-CoA to the loading site and the subsequent movement of the C2 and C3 units attached to the central ACP-like protein, which serves as the substrate component for the peripherally oriented enzyme systems. *(Adapted from Collomb* et al., *2006.)*

The mechanism involved in chain length termination, particularly in the synthesis of short- and medium-chain fatty acids, remains unclear. Smith (1980) examined the mechanism of chain length termination in milk fat synthesis by selectively cleaving the thioesterase component from the fatty acid synthetase complex. This was based on previous studies which showed that limited proteolysis of the fatty acid synthetase complex from rat liver and mammary gland with chymotrypsin and trypsin produced defective chain termination (Agradi *et al.*, 1976). Trypsin was later shown to selectively cleave the thioesterase component of the fatty acid synthetase complex (Smith *et al.*, 1976; Dileepan *et al.*, 1978). The fatty acid synthetase complex appeared to be composed of two identical subunits, each susceptible to tryptic attack at three sites. Thioesterase I occupied a terminal locus at one end of the two polypeptide chains. It had a molecular weight of 25,000 with a trypsin-susceptible site near the center. The core of the fatty acid synthetase complex remaining after the addition of trypsin retained the 4′-phosphopantatheine moiety as well as the enzyme activities associated with it, with the exception of thioesterase. The complex was still capable of synthesizing long-chain fatty acids but now lacked the ability to terminate this process. Related studies using an inhibitor of thioesterase I activity also showed the ability of the modified multienzyme complex to synthesize a single acyl moiety. Unlike the dimer, fatty acid synthetase monomer catalyzed all of the reaction described previously with the exception of condensation, which is only carried out by the former (Kumar *et al.*, 1970). The separation of the components of fatty acid synthetase involved in chain growth and chain termination provided a unique opportunity to pinpoint chain length regulation. Since fatty acid synthetase from animal tissues preferentially synthesizes C16:0, a common mechanism must be responsible for the termination of the acyl chain. Thus, removal or inhibition of thioesterase I resulted in the modified multienzyme complex elongating the fatty acid chain to C22; however, the rate of elongation of C16:0 to C18:0 was much slower than the rate of formation of C16:0, with further chain elongation being even slower. If thioesterase I was involved in chain length determination, there would be little activity toward short- and medium-chain thioesters, with a sharp distinction between C14 and C16 thioesters. The enzymes from liver and mammary multienzymes were identical, with a preference for long-chain C16 and C18 acyl moieties.

Studies on the specificity of the condensing enzyme, the chain-elongating enzyme, and the chain-terminating enzyme (thioesterase I) pinpointed where chain lengthening was controlled. The inability of the condensing enzyme to elongate rapidly beyond C16, and of thioesterase I to hydrolyze acyl thioesters shorter than C16:0, ensured that C16:0 would be the main product synthesized. Examination of the great diversity in the fatty acid distribution between species shows that rats, rabbits, rodents, and mice all have large amounts of medium-chain fatty acids, while

guinea-pigs have only long-chain fatty acids in milk fat. Smith (1980) demonstrated the presence of a tissue-specific thioesterase II in the mammary glands of rabbits, rats, and mice, which was responsible for modification of the product specificity of fatty acid synthetase. This enzyme was isolated and purified from rabbit and rat mammary glands and when added to a purified fatty acid synthetase it switched production from C16 to medium-chain fatty acids. It was intimately involved with the fatty acid synthetase complex in ruminant mammary glands.

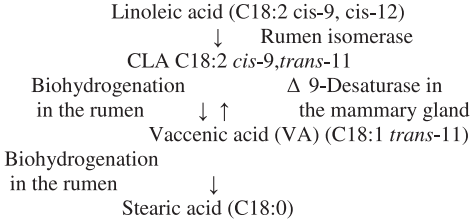
3. Synthesis of Unsaturated Fatty Acids

It is well known that bovine mammary cells actively desaturate C18:0 to C18:1. Subcellular fractionation of lactating and non-lactating cow mammary tissues indicated that the desaturase activity occurred exclusively in the microsomes. Monounsaturated fatty acids, such as oleic acid (C18:1), account for 25% of the fatty acids in milk fat, while polyunsaturated fatty acids are around 2.4% by weight of the total fatty acids. Of these, linoleic acid (C18:2) and α-linolenic acid (C18:3) contributed 1.7% and 0.7%, by weight of the total fatty acids, respectively (Mansson, 2008). In addition, *trans* fatty acids with one or more *trans*-double bonds were reported to account for around 2.7% of the fatty acids in milk (Precht and Molkentin, 1995). These *trans* fatty acids, a mixture of positional and geometric isomers of octadecadinenoic acid containing conjugated double bonds, are referred to as conjugated linoleic acids (CLAs). The main CLA isomer in milk fat, however, is *cis*-9,*trans*-11, accounting for 80−90% of the total CLA (Parodi, 1977; Chin *et al.*, 1992; Sehat *et al.*, 1998). The biosynthesis of CLA in milk fat involves both isomerization and biohydrogenation of unsaturated fatty acids by rumen bacteria as well as Δ^9-desaturase in the mammary gland (Collomb *et al.*, 2006). The main isomer, *cis*-9,*trans*-11 CLA, is formed from linoleic and α-linolenic acids (Bauman *et al.*, 2003). The pathway involved in the biosynthesis of the *cis*-9,*trans*-11 CLA isomer is shown below in Scheme 4.3.

The scheme shows that linoleic acid is rapidly isomerized to CLA *cis*-9,*trans*-11 by *cis*-12,*trans*-11 isomerase and then hydrogenated by rumen bacteria, *Butyrivibrio fibrisolvens*, to vaccenic acid (VA, *trans*-11) (Kepler and Tove, 1967). Loor *et al.* (2002) found a strong correlation between the amount of C18:2 in the diet and the level of vaccenic acid in milk fat. Because VA is hydrogenated at a much slower rate by other microorganisms it accumulates in the rumen (Griinari *et al.*, 1997; Harfoot and Hazelwood, 1997). VA is then converted back to CLA *cis*-9,*trans*-11 by Δ^9-desaturase in the mammary gland (Griinari *et al.*, 2000; Piperova *et al.*, 2002; Mosley *et al.*, 2006). Kraft *et al.* (2003) proposed that CLA (*cis*-9,*trans*-11) is formed indirectly from α-linolenic acid (C18:3) by first being isomerized to a conjugated triene (*cis*-9,*trans*-11,*cis*-15, C18:3) by isomerase in the rumen and then reduced by biohydrogenation to *trans*-11,*cis*-5 C18:2,*trans*-11 18:1 (VA).

B. Milk Proteins

The proteins of milk are a heterogeneous mixture and include two main groups: caseins and whey or serum proteins. These are composed of six major proteins: α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin. Other protein fractions are also present but at very low levels, including bovine serum albumin (BSA), immunoglobulins, lactoferritin (LF), PP3, and ceruloplasmin (Fox and Mulvihill, 1982). Caseins represent the major protein group and were defined by Jenness *et al.* (1956) as phosphoproteins precipitated from raw milk by acidification to pH 4.6 at 20°C. The residual proteins in the serum or whey after the removal of caseins were referred to as whey proteins. The nomenclature of proteins in cow's milk has undergone a number of revisions in response to the improved separation of the proteins by gel electrophoresis. The fifth revision of *Nomenclature of the proteins of cow's milk*, by Eigel *et al.* (1984), recommended that electrophoresis no longer be used as the basis for classification. Instead, they suggested that caseins be identified according to the homology of their primary amino acid sequences into four genetic families: α_{s1}-, α_{s2}-, β-, and κ-caseins. Gel electrophoresis was still useful, however, for identifying the different



SCHEME 4.3 Biosynthesis of CLA isomer *cis*-9,*trans*-11 isomer in milk from C18:2. *(Adapted from Collomb* et al.*, 2006)*

members of these families. Farrell *et al.* (2004), in the sixth revision of *Nomenclature of the proteins of cow's milk*, suggested that a flexible nomenclature system should be adopted that can incorporate new discoveries.

1. Caseins

Caseins account for approximately 80% of the total protein in cow's milk. They are present as macromolecular aggregates or 'casein micelles' ranging in size from 30 to 300 nm. These micelles scatter light and are responsible for the whitish opaque nature of skim milk. The major casein fractions, α_{s1}-, α_{s2}-, κ-, β-, and γ-caseins, account for 38%, 10%, 36%, 13%, and 3% of whole casein, respectively (Davies and Law, 1980). The individual casein fractions differ from each other in their behavior towards calcium ions. Waugh and von Hippel (1956) separated micellar casein into a calcium-sensitive and a calcium-insensitive fraction. This was based on the differential solubilities in the presence of specified amounts of calcium ions, resulting in the identification of two fractions referred to as α_s-casein and κ-casein. Aschaffenburg (1961), using alkaline urea paper electrophoresis, demonstrated genetic variants in cow's milk caseins, thus confirming genetic polymorphism. This was consistent with earlier studies by Aschaffenburg and Drewry (1955), who reported the presence of genetic variants in whey proteins. The three genetic variants for α_{s1}-casein were designated A, B, and C, based on their decreasing mobility during electrophoresis in urea-containing starch gels by Thompson *et al.* (1962). A fourth genetic variant, α_{s1}-D, was identified by Grosclaude *et al.* (1966). Genetic polymorphism occurs as a result of substitution of one or two amino acids in the same protein, although in the case of α_{s1}-D, eight amino acid residues are deleted. This phenomenon is directly related to breed and genus (Aschaffenburg, 1968; Bell *et al.*, 1981; Swaisgood, 1982). With the exception of α_{s1}-D and κ-caseins, differences between genetic variants, however, do not have any technological importance. Variants of β-casein were reported earlier by Aschaffenburg (1961) and designated A, B, and C. A fourth genetic variant, β-casein D, was identified in *Bos indicus* by Aschaffenburg *et al.* (1968).

The κ-casein fraction originally identified by von Hippel and Waugh (1955) and Waugh and von Hippel (1956) was considered to be identical to the protective colloid Z-casein described by Lindstrom-Lang and Kodoma (1925). It was later found that κ-casein was associated with α_s-casein, stabilizing it from being precipitated by calcium ions. This fraction appeared to stabilize the casein micelles as well as limit their size. κ-Casein was also composed of several genetic variants, including A and B (Neeling, 1964; Schmidt, 1964; Woychik, 1964).

2. Molecular and Structural Characteristics of Caseins

The complete amino acid sequence has been determined for the primary structure of all the major bovine caseins (Mercier *et al.*, 1971; Jollès *et al.*, 1972; Ribadeau-Dumas *et al.*, 1972; Grosclaude *et al.*, 1973), which permits their average hydrophobicity to be calculated.

a. α_{s1}-Casein

α-Casein is the largest fraction and includes those phosphoproteins precipitated at low calcium concentrations. The α_{s1}-casein (α_{s1}-CN) family makes up 40% of this fraction and contains 199 amino acid residues, of which 8.4% are prolyl residues evenly distributed throughout the polypeptide chain. A highly charged region is evident between residues 41 and 80, which contains eight very acidic phosphoseryl residues. The rest of the molecule has a net zero charge, although there are three strong hydrophobic regions at 1−40, 90−110, and 130−199 amino acid residues. α_{s1}-Casein is typical of caseins in being an amphiphilic molecule, dominated by an acidic peptide at one end, while the other end is capable of forming hydrophobic bonds. The five genetic variants designated for α_{s1}-casein are A, D, B, C, and E, in order of decreasing relative electrophoretic mobilities in alkaline gels containing urea. Three new genetic variants of α_{s1}-CN F have been reported since the last edition of this book. These include α_{s1}-CN F in German black and white cattle (Erhardt, 1993), α_{s1}-CN G in Italian brown cattle (Mariani *et al.*, 1995), and α_{s1}-CN H in African animals (Mahe *et al.*, 1999). The major genetic variant in *B. taurus* is the B variant. If such differences between genetic variants are due to phosphorylation, this is now designated by an Arabic number and a letter after the Latin letter which indicates the particular genetic variant (Eigel *et al.*, 1984). For example, the main genetic variant B for α_{s1}-casein in *B. taurus* with eight phosphoseryl residues is now designated α_{s1}-CN B-8P (Figure 4.2).

b. α_{s2}-Casein

α_{s2}-Casein (α_{s2}-CN) accounts for 10% of this fraction and is composed of 207 amino acid residues. It is the most hydrophilic of the caseins, with 10−13 phosphoseryl residues located in three charged regions, 8−16, 56−61, and

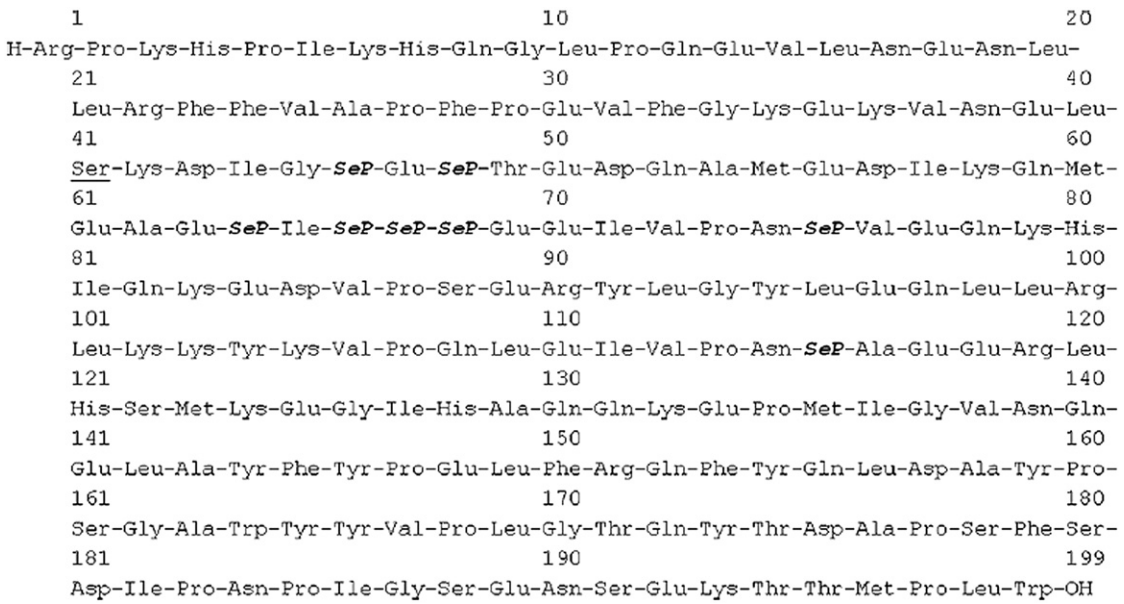


FIGURE 4.2 Primary structure of *Bos* α_{s1}-CN B-8P. *(Mercier* et al.*, 1971; Grosclaude* et al.*, 1973; Stewart* et al.*, 1984; Nagao* et al.*, 1984; Koczan* et al.*, 1991; Farrell* et al.*, 2004)*

129−133. It has a very strong hydrophobic C-terminal region (160−207) and a weak hydrophobic segment between 90 and 120 (Fox and Mulvihill, 1982). α_{s1}-Casein also contains cysteine residue(s), which may be involved in thermally induced thiol−disulfide interactions. Four genetic variants have been reported, designated A, B, C, and D. The milk components previously classified as α_{s3}-, α_{s4}-, α_{s5}-, and α_{s6}-casein (Annan and Manson, 1969; Rose *et al.*, 1970; Whitney *et al.*, 1976) were all components of the α_{s2}-CN family with a similar amino acid sequence differing only in the degree of phosphorylation. Eigel *et al.* (1984) recommended that a tentative nomenclature for the A variant be changed as indicated in Table 4.5. α_{s5}-Casein, however, is a dimer of the α_{s3}- and α_{s4}-caseins, now designated α_{s2}-CN A-12P and α_{s2}-CN A-11P, and joined by a disulfide bond (Hoagland *et al.*, 1971). As a result of cDNA sequencing (Stewart *et al.*, 1987) and genomic DNA sequencing (Groenen *et al.*, 1993), the primary structure of α_{s2}-CN A-11P, originally reported by Brignon *et al.* (1977), has been modified. This is shown in Figure 4.3, where Gln replaces Glu at position 87.

c. β-Caseins

β-Caseins (β-CN) comprise up to 45% of total caseins and are also phosphoproteins. They differ from α-caseins by their strong temperature-dependent association as well as the temperature dependency of their solubility in the presence of calcium ions. β-Casein has 209 amino acid residues, of which 16.7% are proline evenly distributed along the polypeptide, which limits the formation of an α-helix. Seven genetic variants are recognized, which separate differently by electrophoresis, depending on whether acidic or alkaline conditions are used. While A can be differentiated from B, C, and D by electrophoresis under alkaline conditions, the A variants can only be separated under acidic conditions. Details on the nature and nomenclature of these variants were reviewed by Eigel *et al.* (1984). Since

TABLE 4.5 Recommended Changes for Nomenclature of the α_{s2}-CN A Family

Former Nomenclature	Recommended Nomenclature
α_{s2}-CN A	α_{s2}-CN A-13
α_{s3}-CN	α_{s2}-CN A-12
α_{s4}-CN	α_{s2}-CN A-11
α_{s6}-CN	α_{s2}-CN A-10

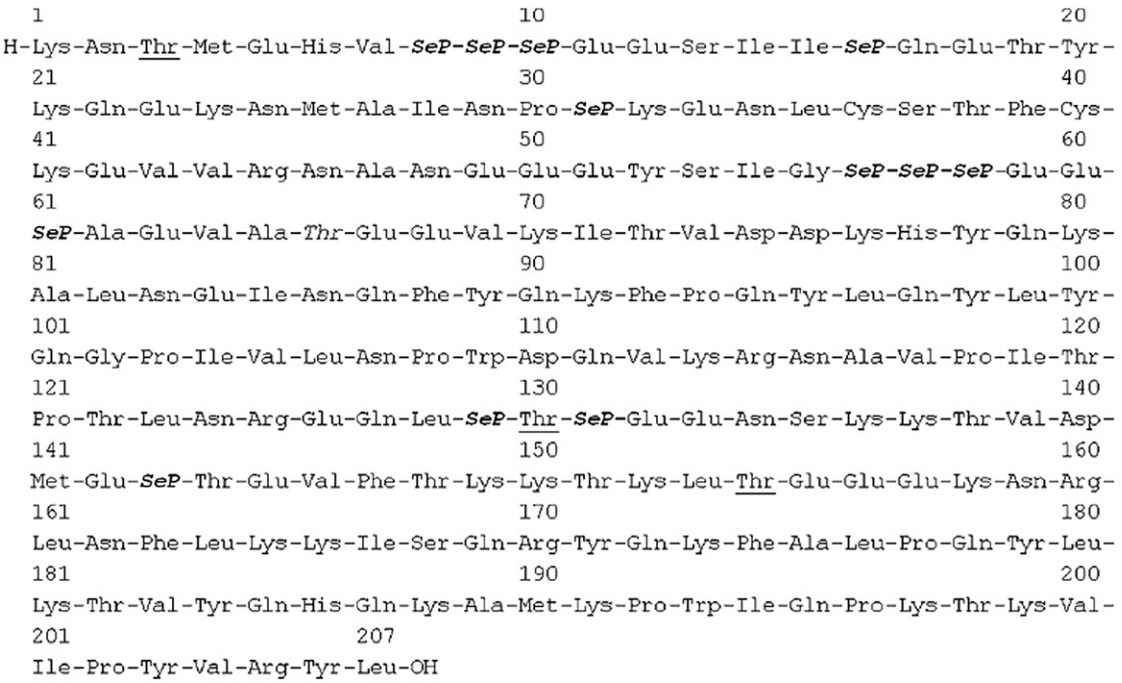


FIGURE 4.3 Primary structure of *Bos* α_{s2}-CN A-11P (Brignon *et al.*, 1977; Mahe and Grosclaude, 1982; Stewart *et al.*, 1987; Groenen *et al.*, 1993). Seryl residues (SeP) identified as phosphorylated are indicated in italic, bold type *(Farrell* et al.*, 2004).*

then, three new variants were identified by sequence as β-CN F, formerly known as β-CN X (Visser *et al.*, 1995), β-CN G (Dong and Ng-Kwai-Hang, 1998), and β-CN H (Han *et al.*, 2000).

The primary sequence for the most common A2 variant, β-CN A2-5P, is shown in Figure 4.4. β-Caseins are the most hydrophobic of the casein fractions because of the number of hydrophobic residues present, although they have a strongly charged N-terminal region. Techniques including circular dichroism and spherical rotary dispersion

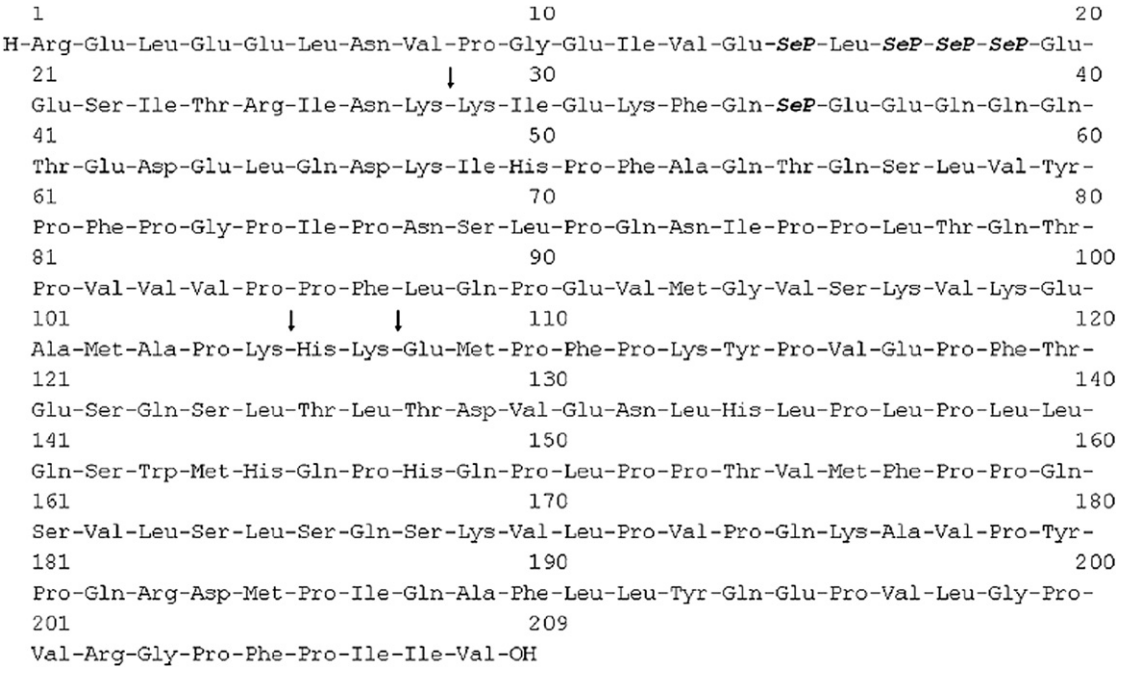


FIGURE 4.4 Primary structure of *Bos* β-CN A^2-5P. *(Ribadeau-Dumas* et al.*, 1972; Grosclaude* et al.*, 1973; Farrell* et al.*, 2004)*

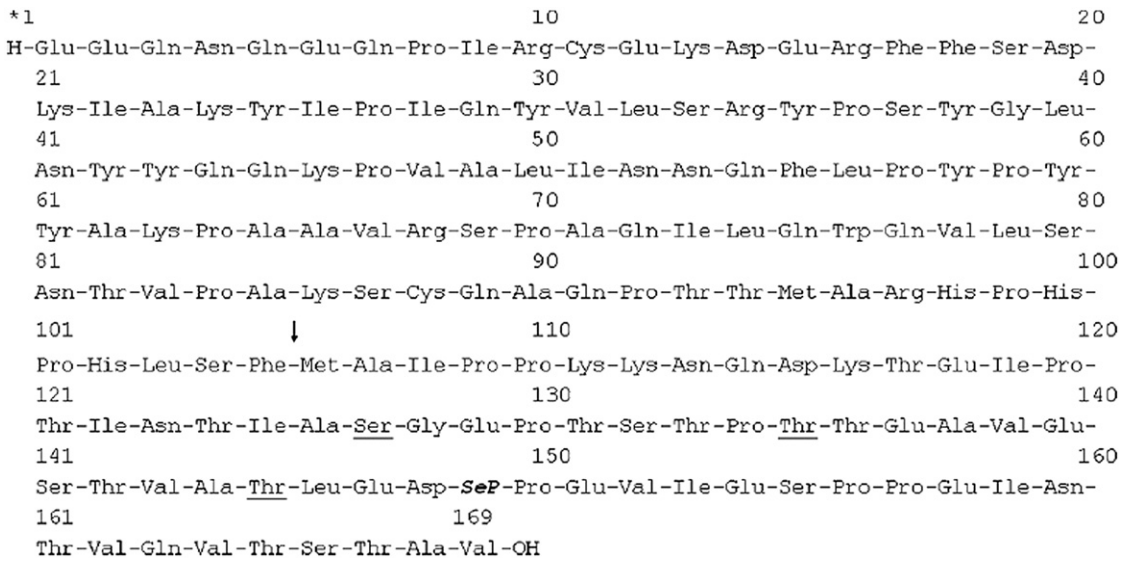


FIGURE 4.5 Primary structure of *Bos* κ-CN B-1P (Mercier *et al.*, 1973). The arrow indicates the point of attack by chymosin (rennin). The asterisk (*) indicates pyroglutamate as the cyclized N-terminal. The site of post-translational phosphorylation (SeP) is indicated in italic, bold type; residues that may potentially be phosphorylated are underlined *(Farrell* et al.*, 2004).*

excluded the presence of secondary and tertiary structures, although Andrews *et al.* (1979) calculated the presence of 10% α-helix, 13% sheets, and 77% unordered structure in β-casein.

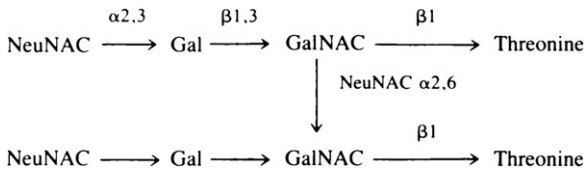
d. γ-Casein

At one time γ-casein was considered to be a distinct fraction accounting for 3% of whole casein. It was shown by electrophoresis to be identical to the C-terminal portion of β-casein (Gordon *et al.*, 1972; Groves *et al.*, 1973). Trieu-Cuot and Gripon (1981), using electrofocusing and two-dimensional electrophoresis of bovine caseins, obtained two-dimensional patterns by enzymatic hydrolysis of β-casein with bovine plasma similar to γ-casein. Studies conducted by a number of researchers suggested that β-casein was hydrolyzed by milk proteinase (plasmin) at three sites adjacent to lysyl residues 28, 104, and 106, producing six polypeptides including γ_1, γ_2, and γ_3-caseins, as well as protease peptones (heat-stable, acid-soluble phosphoproteins) found in milk (Groves *et al.*, 1973; Eigel, 1977; Andrews, 1979). Thus, γ-casein could arise by trypsin-like proteolysis of β-casein before or after milking. The fifth revision of the 'Nomenclature of the proteins of cow's milk' no longer categorized γ-caseins but considered them degradation products of β-casein (Eigel *et al.*, 1984). In the sixth edition, by Farrell *et al.* (2004), the plasmin cleavage products of β-CN were identified as γ_1, γ_2, and γ_3 CN variants and proteose peptones.

The open structure and hydrophobicity of caseins render them extremely susceptible to proteolysis, with a high propensity to formation of bitter peptides (Guigoz and Solms, 1976). Caseins are accessible to attack by the indigenous milk or psychotrophic proteinases, which do not affect the whey proteins (Fox, 1981).

e. κ-Casein

κ-Casein (κ-CN) contains 169 amino acid residues, of which 11.8% are proline (Figure 4.5). The major κ-CN component is carbohydrate free while the minor κ-CN, a glycoprotein, is thought to be glycosylated forms of the major κ-CN. The carbohydrate portion contains N-neuraminic acid (NANA), galactose (Gal), and *N*-acetylgalactosamine (NeuNAC), and is present as either a trisaccharide or a tetrasaccharide (Jollès and Fiat, 1979). The points of attachment between colostral κ-CN and the complex oligosaccharides were shown to be threonine (Thr) residues 131, 133, and 135. Saito and Itoh (1992) confirmed the structures of these carbohydrate moieties and identified three more. The lack of information regarding the structure of the minor κ-CN components made nomenclature of these casein fractions inconclusive in the fifth revision (Eigel *et al.*, 1984). In the sixth revision, Farrell *et al.* (2004) still precluded drawing up a precise nomenclature for these minor κ-casein fractions owing to their high degree of heterogeneity and the limited amounts of κ-CN.



κ-Casein, an amphiphilic molecule with only one phosphoseryl residue, has charged trisaccharide or tetrasaccharide moieties located in the C-terminal segment. The rest of the molecule, however, is highly hydrophobic in character. This protein is unique in that it is soluble in calcium solutions that would normally precipitate the other casein fractions. κ-Casein exerts a stabilizing effect on the casein fractions by forming colloidal micelles. It is this protein fraction that is specifically hydrolyzed by rennin, which releases a macropeptide from the C-terminal region containing the carbohydrates. The specific bond hydrolyzed in κ-casein is 105−106, the Phe−Met linkage. The remaining product with the N-terminus and two-thirds of the original peptide chain is referred to as para-κ-casein. This reaction, discussed in Chapter 8, destabilizes the casein micelle, causing formation of the curd.

3. Whey Proteins

Whey, the major coproduct of the cheese and casein industries, was considered at one time to be a waste product by cheese makers and casein manufacturers (Smithers, 2008). However, it is now considered to be a functional food because of the bioactive properties that whey proteins and individual fractions exhibit (Kruger *et al.*, 2005; Michaelidou and Steijns, 2006). It contains a group of proteins that remain soluble in the milk serum or whey following the precipitation and removal of the caseins at pH 4.6 and 20°C. The major whey proteins, β-lactoglobulin (β-LG), and α-lactalbumin (α-LA), are globular proteins that account for 70−80% of the total whey proteins (Chatterton *et al.*, 2006). The other minor fractions include lactoferrin and lactoperoxidase.

a. β-Lactoglobulins

β-LGs normally provide immunological protection and, because of this and other properties, they are now highly valued as nutraceuticals. They are present as genetic variants, with A and B variants found most frequently in most breeds of cows. The A variant is expressed at a higher level than either the B (Aschaffenburg and Drewry, 1955) or C variant (Hill *et al.*, 1996; Ng-Kwai-Hang and Grosclaude, 2003). Additional variants have also been reported, including H, I, J, and W. The primary sequence of β-LG B is shown in Figure 4.6 and contains 162 amino acids (Farrell *et al.*, 2004).

b. α-Lactalbumin

α-LA normally accounts for 3−4% of the total protein in milk or 20% of the whey proteins (Swaisgood, 1995). It is a globular protein containing 123 amino acids (Brew *et al.*, 1970) with a high degree of homology between the α-LA of other animals including human milk. Together with $\beta_{1,4}$-galactosyltransferase it forms the lactose synthase complex. As discussed later, it facilitates the formation of lactose from glucose and UDP galactose. Two genetic variants are predominant in the mature protein α-LA, A and B (Jenness, 1974), with a third variant C reported, but not yet confirmed, by DNA or protein sequencing (Bell *et al.*, 1981). It is higher in human milk than cow's milk, suggesting that infant formula could be enriched with α-LA whey proteins (Chatterton *et al.*, 2006). The primary structure for α-LA is shown in Figure 4.7.

c. Lactoferritin

LF, the major member of a family of proteins with specific iron-binding properties, is found in the milk of most species (Schanbacher *et al.*, 1993). It occurs as a single polypeptide chain containing 689 amino acids with varying degrees of glycosylation. LF provides the first line of defense against infection and inflammation (Ward *et al.*, 2002), and also displays antiviral activity (Van de Strate *et al.*, 2001).

d. Bovine Serum Albumin

BSA accounts for around 8% of the total whey proteins in milk and is physically and immunologically identical to blood serum albumin (Coulson and Stevens, 1950; Polis *et al.*, 1950). It contains 583 amino acids and 17 disulfide

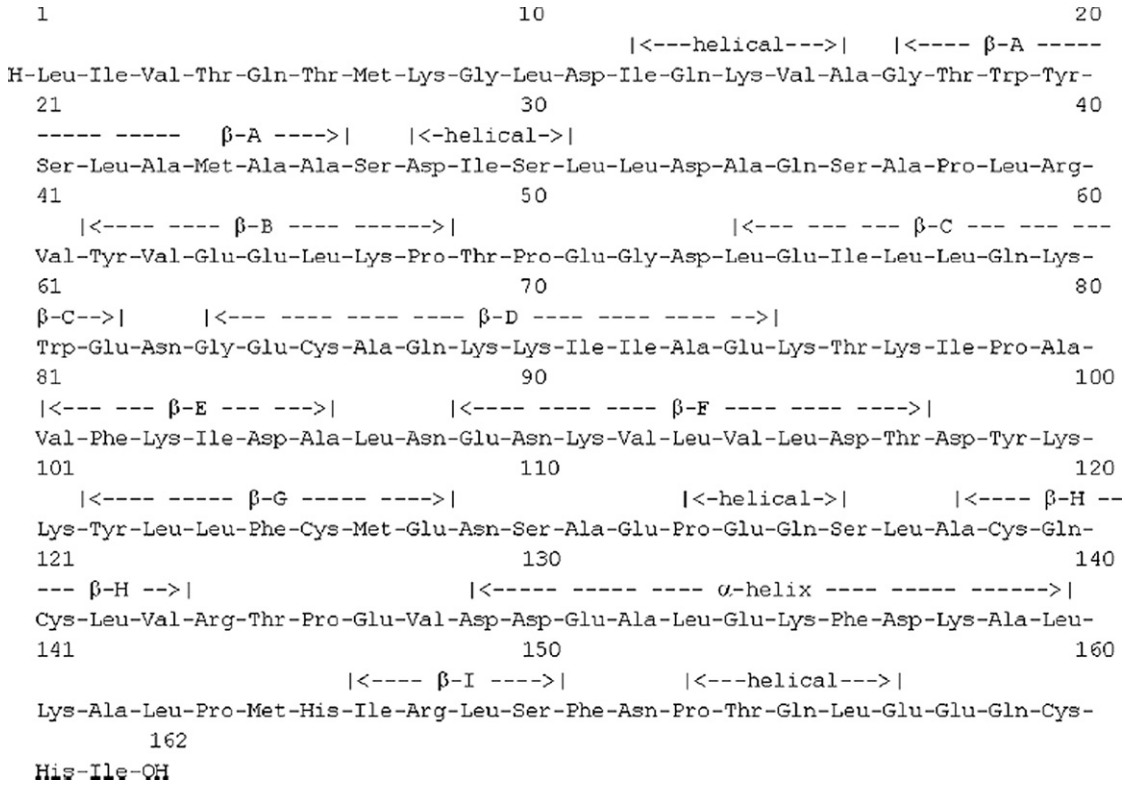


FIGURE 4.6 Primary structure of *Bos* β-LG B (Eigel *et al.*, 1984). The free sulfhydryl groups are on Cys121 in the native form of the protein (Bewley *et al.*, 1997; Brittan *et al.*, 1997; Brownlow *et al.*, 1997; Quin *et al.*, 1998a, b, 1999). The sequence positions of the major secondary structural features, α-helix, helical regions, and β-strands (β-A to β-I) are shown above the main sequence *(Farrell* et al.*, 2004).*

bonds (Hirayama *et al.*, 1990). It plays an important role in the transport, metabolism, and distribution of ligands (Carter and Ho, 1994) as well as in free radical protection.

C. Casein Micelle

The casein proteins in milk are found in a colloidal particle called the casein micelle. These colloidal particles, along with the fat globules, comprise the dispersed phase in milk, surrounded by the serum phase, which consists of lactose, the whey proteins, and dissolved minerals and vitamins. Micelle sizes are polydisperse and can be fitted to a log normal size distribution ranging from 50 to 400 nm with the peak centered at 200 nm, as determined by sedimentation

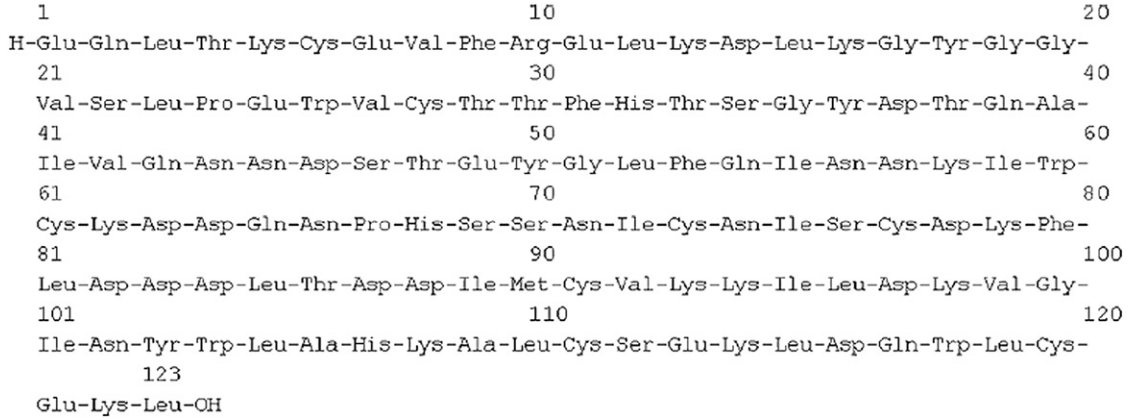


FIGURE 4.7 Primary structure of *Bos* α-LA B (Brew *et al.*, 1970; Vanaman *et al.*, 1970). The disulfide bridges in the molecule are between positions 6 and 120, 28 and 111, 61 and 77, and 73 and 91 *(Farrell* et al.*, 2004).*

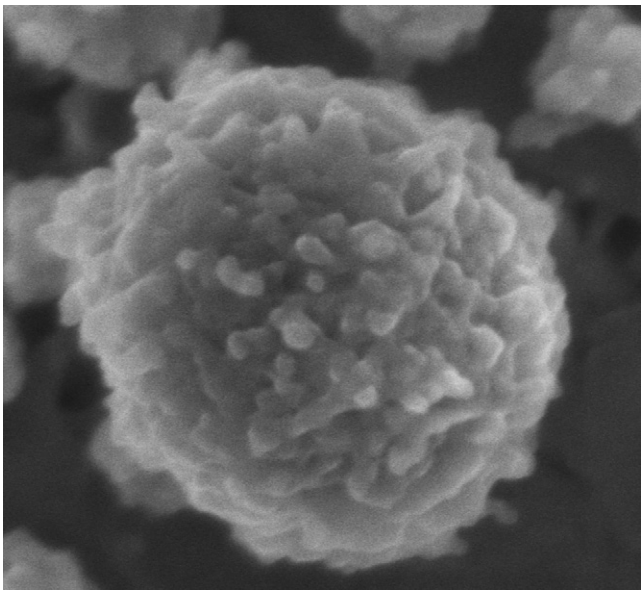


FIGURE 4.8 Image of a casein micelle from field-emission scanning electron microscopy. See Dalgleish *et al.* (2004) for methodology. Bar = 150 nm. *(Courtesy of Professor D. Goff, University of Guelph.)*

field flow fractionation and dynamic light scattering (de Kruif, 1998). Molecular weight determinations suggest that individual micelles could contain as many as 20,000 individual protein molecules (de Kruif and Holt, 2003). In addition to protein (94%), the micelles contain small ions such as calcium, phosphate, magnesium, and citrate, referred to as colloidal calcium phosphate (CaP). These micelles have an open, porous structure, as seen by electron microscopy (Figure 4.8). They are highly hydrated, with a voluminosity of 2−4 ml/g. The stability of the micelles is particularly important as they exert a great influence on the processing properties of the milk. This has resulted in a number of studies on the nature of protein−protein and protein−ion interactions within the micelle structure. The micelle is extremely stable under some conditions of processing, e.g. concentration, ultrafiltration, pelleting, and drying, while being very unstable under others, e.g. acidification and renneting (Horne, 1998). The intricacy of the micelle structure may be related to its biological function in milk, to carry a large amount of highly insoluble CaP to the mammalian young in liquid form, and to form a clot in the stomach for more efficient nutrition (de Kruif and Holt, 2003).

Numerous models of the surface and internal structure of casein micelles have been proposed over a period of more than 50 years, including the core coat, chain polymer, and submicelle models (Waugh and Noble, 1965; Payens, 1966, 1979; Garnier and Ribadeau-Dumas, 1970; Waugh *et al.*, 1970; Schmidt and Payens, 1976; Slattery, 1976; Schmidt, 1980; McMahon and Brown, 1984; Walstra, 1990, 1999). Current models include the CaP nanocluster model (Holt *et al.*, 1998, 2003; de Kruif and Holt, 2003), the dual-binding model (Horne, 1998, 2002, 2006), and the interlocking lattice model (McMahon and Oommen, 2008). With all of the available information and techniques that have been applied to studying casein micelles, it appears now that these models all differ only in fine detail but the essential elements of casein structure have been agreed upon. The reader is referred to a number of recent references for a complete discussion of casein micelle structures (de Kruif and Holt, 2003; Dalgleish *et al.*, 2004; Horne, 2006; McMahon and Oommen, 2008).

1. Casein Micelle Structure

About 75−80% of the proteins in milk are classed as casein protein, that which precipitates at pH 4.6. Most, but not all, of this casein protein exists in the casein micelle, which contains other components as well as casein, including calcium, phosphate, citrate, minor ions, lipase and plasmin enzymes, and entrapped milk serum. This particle is a casein protein:calcium-phosphate complex, and not a true micelle in the colloidal sense. The molar ratio of proteins within the micelle at the time of secretion is approximately α_{s1}: α_{s2}: β: κ = 4:1:3.5:1.5, or about 38% α_{s1}-, 10% α_{s2}-, 36% β-, and 13% κ-casein. α_s- and β-caseins are extensively phosphorylated on serine residues and will precipitate in the presence of calcium ions or CaP, whereas κ-casein is not sensitive to calcium precipitation. Owing to the high

concentration of proline residues, α_s- and β-caseins have been referred to as rheomorphic, lacking a specific secondary structure. They also have high hydrophobicity (Walstra *et al.*, 2006).

The κ-casein content requires special consideration. It plays a unique role during gelation of milk by chymosin in that it is cleaved by chymosin at the 105−106 Phe−Met bond into para-κ-casein (residues 1−105) and the hydrophilic caseinomacropeptide (residues 106−169) fractions. An inverse relationship between κ-casein content and micelle size has been reported (Schmidt, 1979; McGann *et al.*, 1980; Donnelly *et al.*, 1984). Micelles in the 154 nm diameter range may contain as little as 4% κ-casein whereas those in the 62 nm diameter range contain up to 12% κ-casein (Donnelly *et al.*, 1984). Decreases in α_{s1}-, α_{s2}-, and β-casein were noted as κ-casein increased. Artificial micelles can be prepared if α_{s1}- or β-casein is absent, but not if κ-casein is absent (Schmidt, 1980). Thus, it plays an important role in casein micelle stabilization. The macropeptide portion, once cleaved by chymosin, is readily soluble and migrates away from the micelle. The para-casein micelle, devoid of the negatively charged caseinomacropeptide, coagulates readily with other para-casein micelles forming curd. Therefore, κ-casein must exist at or near the surface of the micelle, orientated within easy access of the caseinomacropeptide to cleavage by chymosin. This macropeptide is thought to exist in a hairy manner covering the outer surface of the micelle (Walstra, 1979; Walstra *et al.*, 1981; Holt and Dalgleish, 1986; Holt and Horne, 1996) and stabilizing it by a combination of electrostatic and steric mechanisms.

About 7% of the dry matter of the micelles consists of inorganic material, principally calcium and phosphate (Holt *et al.*, 1998). Milk contains approximately 117 mg calcium per 100 g of milk. Milk serum contains 40 mg per 100 g of serum, only 32% of the calcium content. The rest is associated with the casein micelle, approximately 31 mg per gram of dry casein. Likewise, of 203 mg of inorganic phosphate per 100 g of milk, only 53% is present in the serum and the rest, 37 mg per gram of dry casein, is associated with the micelle. The micelle also contains 5.6 mg citrate, 3.3 mg K, 1.5 mg Mg, and 0.9 mg Na per gram of dry casein (Walstra *et al.*, 2006).

The micelles may contain as much as 7.9 g of water (Farrell *et al.*, 1990), or milk serum depleted of large solute molecules such as globular proteins (Walstra *et al.*, 2006), per gram of protein. The micelle is more highly solvated than most globular proteins and therefore has a rather porous structure. The voluminosity of the micelles ranges from 2 to 4 ml per gram of casein, depending on how far one considers the outer hairy layers of the micelle to extend, representing 6−12% of the volume fraction of milk (Walstra, 1979).

Many studies on the size distribution of the micelles have been conducted using electron microscopy, light scattering, and controlled pore glass chromatography methods. The average diameter has been reported as 25−140 nm, and the volume surface average diameter as 86 nm (Schmidt and Payens, 1976). Micelle sizes can be fitted to a log normal size distribution ranging from 50 to 400 nm with the peak centered at 200 nm, as determined by sedimentation field flow fractionation and dynamic light scattering (de Kruif, 1998), although results vary according to the technique used. A small number of very large particles, up to 800 nm in diameter, and a large number of small particles have been reported (Walstra *et al.*, 2006).

The molar ratios of the casein fractions of the micelle given above refer to the micelle at the time of secretion. However, it has been recognized that β-casein is able to migrate out of the micelle to the serum phase in a reversible manner without causing collapse of the micellar structure (Creamer *et al.*, 1977; Ali *et al.*, 1980). This migration is temperature dependent. As much as 60% of the β-casein has been found in the serum phase after 48 hours at 4°C. This serum β-casein is free to interchange with micellar casein. These changes, however, are reversible upon rewarming to 37°C. This cold dissociation phenomena has a pedagogical impact on a model for the casein micelle, as it suggests the importance of hydrophobic interactions for intramolecular interactions in the interior of the micelle, and also has a technological impact, particularly on the cheese industry, since cheese-making parameters may be altered (Ali *et al.*, 1980) and enhanced proteolysis of the β-casein by plasmin and proteinases of the bacterial microflora may occur in the serum phase (Fox and Guiney, 1973; Creamer *et al.*, 1977).

2. Casein Micelle Models

All of the above considerations have led to numerous proposed models for the casein micelle, which have evolved and been continually refined with new experimental techniques and results. For many years, the most accepted model was the submicelle model elaborated by Schmidt (1980) and refined by Walstra (1990, 1999). The strongest evidence for submicelles of 8−20 nm in diameter came from the porous nature of the micelle, elaborated by early electron microscopy techniques as having a raspberry-like appearance (Schmidt and Buchheim, 1970, 1976). The submicelles were suggested to contain a hydrophobic core (Farrell *et al.*, 1990) covered by a hydrophilic coat much less dense

than globular proteins (Farrell *et al.*, 1990), which is at least partly comprised of the polar moieties of κ-casein (Walstra, 1990). The κ-casein formed hair-like projections from the surface of the micelle, with an effective thickness of the hairy layer of least 7 nm (Walstra, 1990).

The submicelle model called for two types of micelles, those rich in κ-casein at the surface and those not. Submicelles were suggested to be aggregated by CaP until such time as the entire surface was coated with κ-casein, thus containing hundreds of submicelles held together by CaP. This, in fact, is the great difficulty with this model: there is no inherent reason why, during the synthesis of caseins and casein micelles in the secretory cell, there would be two types of submicelles of differing composition, nor have any such submicelles ever been found (McMahon and McManus, 1998; Holt *et al.*, 2003; Dalgleish *et al.*, 2004).

Association of casein proteins begins in the Golgi vesicles within the secretory cell. The fully phosphorylated but non-aggregated caseins leave the Golgi apparatus in these vesicles. Calcium and phosphate pass through the membrane into the Golgi vesicles, and when their concentration exceeds the solubility of CaP, micellar aggregation begins to occur. When the Golgi vesicles fuse to the apical plasma membrane, intact micelles are emptied into the alveolar lumen (Farrell *et al.*, 2006).

It must be remembered that one of the biological roles of the casein micelle is to carry much higher concentrations of bioavailable calcium to the infant mammalian than can be carried in solution. The model proposed by Holt *et al.* (1998, 2003) described casein micelles as a homogeneous network of casein polymers containing nanoclusters of CaP. In this model, the biological role of the micelle is addressed, as it has been shown that the phosphoserine residues of α_s- and β-caseins can prevent CaP from precipitating once the association of calcium and phosphate during biosynthesis exceeds solubility (Holt *et al.*, 1998). Hence, it is the biological role of the caseins to sequester CaP into a colloidal particle. The CaP—casein nanocluster, then, resembles to some extent the older casein submicelle, but it has been derived from a better understanding of the biosynthesis process. The dual-binding model of Horne (1998, 2002, 2006) emphasizes the dual abilities of the various casein proteins, except for κ-casein, either to self-associate or to associate with CaP. It is the self-association of caseins that begins to aggregate the nanoclusters. During biosynthesis, formation and aggregation of nanoclusters continue, but κ-casein association with either β- or α_s-casein acts as a chain terminator, which eventually leads to a surface coverage of κ-casein and cessation of aggregation. This evolves the role of κ-casein from the previous submicellar models, as it explains how κ-casein ends up forming the surface hairy layer and it eliminates the need for two different populations of submicelles, as discussed above. Marchin *et al.* (2007), using small-angle X-ray scattering analysis, also concluded that casein micelles were likely to consist of a complex network of protein chains. The only particulate substructures present were considered to be CaP nanoclusters.

McMahon and Oommen (2008) have brought the ideas of the nanocluster model and the dual-binding model together to elaborate what they see as an interlocked lattice type of supramolecular structure, which includes both protein chains and protein—CaP nanoclusters. In their study, they immobilized casein micelles on poly-L-lysine and parlodion-coated copper grid with proteins and CaP stained with uranyl oxalate. Their protocol included instantaneous freezing and drying under high vacuum to obtain stereo images by high-resolution transmission electron microscopy (TEM). These, together with the cross-sectional freeze-fracture replica images produced by TEM by Heertje *et al.* (1985) and Karlsson *et al.* (2007) and the field-emission scanning electron microscope images of Dalgleish *et al.* (2004), provide perhaps the best structural representations to date. Based on these images, McMahon and Oommen (2008) proposed the supramolecular structure shown in Figure 4.9, as an interlocked lattice. The proteins appear as spheres of 8 nm in diameter that both surround CaP nanoclusters and extend as short chains between the interlocking points and out from the periphery. The overall structure is irregular and can accommodate a large diversity of linkages among the proteins such as chain extenders (β-casein or α_{s1}-casein), chain branch points (α_{s1}-casein or α_{s2}-casein), chain terminators (κ-casein), and interlocking points (CaP nanoclusters). The supramolecular structure proposed allows for a very stable colloidal particle composed of thousands of protein molecules and hundreds of CaP nanoclusters. The distance between interlocking sites was in agreement with the 19 nm interval for CaP nanoclusters proposed by de Kruif and Holt (2003). The supramolecular model also allowed for the predominance of κ-casein as terminal molecules on the periphery with protuberances that extended into the surrounding environment. This description, in effect, reconciles the nanocluster and dual-binding models into what seems to be a coherent whole.

The surface of the micelle deserves a little more discussion. It is clear that κ-casein plays a vital role in stabilizing the micelle and exists with its macropeptide extending into the serum. However, the surface is penetrable by chymosin to the Phe—Met bond of κ-casein, by β-lactoglobulin during high-temperature heating as it is known to interact

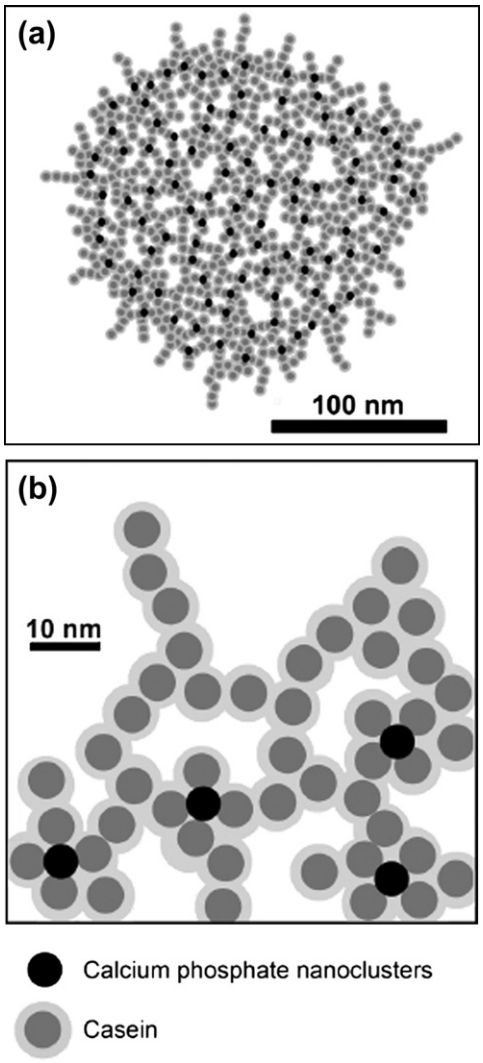


FIGURE 4.9 Schematic diagram of an interlocking lattice model of the casein micelle with calcium phosphate aggregates throughout the entire supramolecule and chains of proteins extending between them. Drawn as cross-sectional scaled views of (a) the complete supramolecule, and (b) a portion of the supramolecule periphery. Calcium phosphate nanoclusters are shown with a diameter of 4.8 nm and approximately 18 nm apart; the caseins are shown with a hydrodynamic diameter of 8 nm *(McMahon and Oommen, 2008)*.

with κ-casein at the surface of the micelle (Diaz *et al.*, 1996), and also by large polymers such as negatively charged κ-carrageenan (Spagnuolo *et al.*, 2005). It must also allow for β-casein to dissociate reversibly during cold aging. In addition, calculations suggest that there is not sufficient κ-casein to cover the entire surface (Dalgleish, 1998). Hence, this implies that it is not a 'hairy tennis ball' (Holt and Dalgleish, 1986; de Kruif, 1998) type of surface structure but rather that it must be distributed unevenly, leaving large gaps or pores in the surface, perhaps at the end of microtubules as described by Dalgleish *et al.* (2004).

D. Biosynthesis of Milk Proteins

The major portion of milk proteins is synthesized by highly specialized mammary secretory cells under genetic control. These produce highly specific proteins that are unique to lactation. The starting materials are free amino acids absorbed from the bloodstream via the basal membrane by a process that involves active transport (Christensen, 1975). The possible role of the γ-glutamyl peptidase cycle in mammary amino uptake was suggested by Baumrucker and Pocius (1978). The enzyme involved, glutamyl transpeptidase (EC 2.3.2.2), catalyzes the transfer of the γ-glutamyl residue from glutathione and/or other γ-glutamyl components to amino acids or peptides (Meister

et al., 1976). This enzyme is thought to regulate cellular glutathione and amino acid transport via the γ-glutamyl cycle:

$$\begin{array}{ccc} \text{glutathione} & + & \text{amino acid} \\ & \downarrow & \\ \gamma\text{-glutamyl-AA} & + & \text{cysteinyl glycine} \end{array}$$

The enzyme γ-glutamyl transpeptidase is secreted into the milk where it associates with the milk membranes, including the MFGM or another membrane obtained from skim milk. The latter is derived from plasma membranes, Golgi apparatus, endoplasmic reticulum, and secretory vessels (Kitchen, 1974). Pocius *et al.* (1981) noted that the level of glutathione was extremely low in the plasma of lactating Holstein cows compared to the level in the blood, where it was 200-fold higher. From *in vitro* studies these researchers found that when arteriovenous differences for free amino acids in plasma were quantitated, there was an apparent shortage of cysteine for milk protein synthesis. The uptake of glutathione by the mammary gland, however, was more than sufficient to account for any cysteine secreted in milk. Pocius and Baumrucker (1980) studied the *in vitro* uptake pattern of nine essential amino acids by mammary slices compared to the known *in vivo* uptake pattern of the same amino acids in the cow's udder. A significant linear correlation ($r = 0.91$) was evident between these patterns of uptake in spite of the many assumptions made (Figure 4.10).

The biosynthesis of milk proteins is similar to other systems in which the genetic message is transmitted from DNA to messenger RNA and then translated at the ribosomal level into the amino acid sequence of polypeptides. Following synthesis, the export milk protein leaves the ribosomes on the outer surface of the rough endoplasmic reticulum and is transported to the Golgi apparatus, where alteration of some of the export protein and synthesis of other major constituents of milk occur.

E. Lactose

The major carbohydrate of milk is lactose, a disaccharide of galactose and glucose linked by an α-(1→4) glycosidic bond. It is sometimes referred to as the milk sugar and accounts for 2% of normal cow's milk. Its complete name is lactose α-(1→4)galactosylglucopyranose.

The biosynthesis of lactose is catalyzed by the enzyme lactase synthetase in which glucose acts as the galactosyl acceptor (Watkins and Hassid, 1962). This enzyme is located in the luminal face of the Golgi dicytosome membrane, where it receives both glucose and UDP-galactose from the cytosol. The reactions involved are shown in Figure 4.11, in which the uridine nucleotide cycle appears to functionally link the two regions (Kuhn *et al.*, 1980). The transfer of glucose, UDP-galactose, and UMP through the Golgi membrane is probably facilitated by a specific carrier in the membrane. The formation of UMP by the enzyme nucleotide diphosphatase permits the removal of UDP released in the

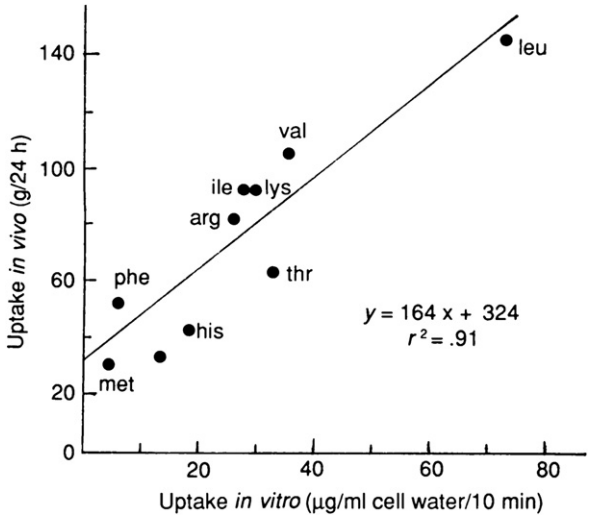


FIGURE 4.10 Relationship between amino acid uptake as measured *in vitro* (abscissa) and *in vivo* (ordinate) (Pocius and Baumrucker, 1980). *(Calculated from data by Clark* et al.*, 1975.)*

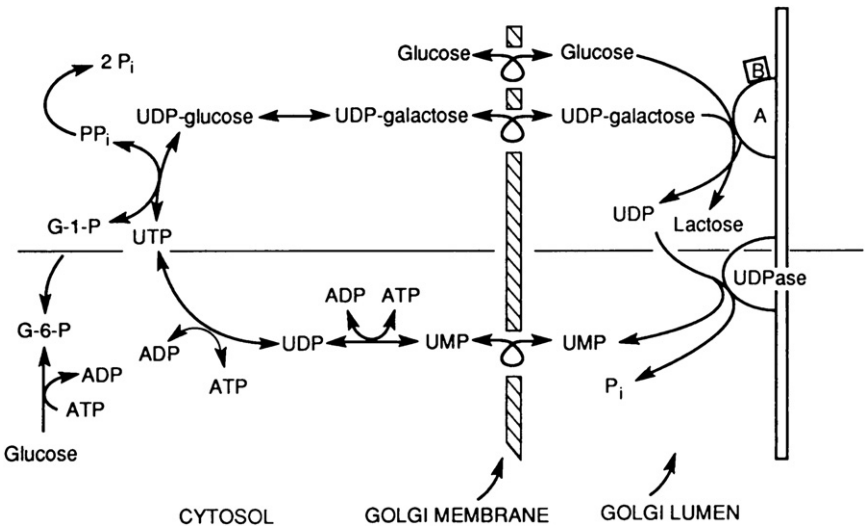


FIGURE 4.11 Uridine nucleotide cycle, supporting lactose synthesis and functionally linking the cytosol and Golgi lumen compartments of the mammary secretory cell. A = Galactosyl transferase; B = α-lactalbumin *(Kuhn* et al., *1980).*

lactose synthetase reaction. This is important as UDP competitively inhibits the lactose synthetase enzyme to form UDP galactose (Kuhn and White, 1975, 1976, 1977). The major steps involved in the biosynthesis of lactose are as follows:

(1) $\text{UTP} + \text{glucose-1-P} \xrightarrow{\text{UDPG-pyrophosphorylase(EC 2.7.79.)}} \text{UDPG-glucose} + \text{PP}_\text{i}$

(2) $\text{UDP-glucose} \xrightarrow{\text{UDPG-galactose-4-epimerase(EC 5.1.3.2)}} \text{UDPG-galactose}$

(3) $\text{UDP-galactose} \xrightarrow{\text{Lactase synthetase(EC 2.6.1.22)}} \text{lactose} + \text{UD}$

Kuhn *et al.* (1980), from their studies on rat mammary glands, summarized the benefits accrued by the compartmentalization of the lactose synthetase system. The free energy involved could be used to synthesize lactose without creating osmotic problems for the rest of the cells. The concentration of UDP-glucose in the cytosol must not exceed that of UDP-galactose by a factor of three. UDP-glucose must itself be prevented from entering the Golgi lumen as it would inhibit lactose synthetase. The separation of nucleoside diphosphatase prevents hydrolysis of diphosphate in the cytosol and the subsequent depletion of phosphate energy. The role of α-lactalbumin (α-LA) in the biosynthesis of lactose was discovered by Brodbeck and Ebner (1966), who found that lactose synthetase was composed of two components, an 'A' protein and an 'R' protein. The A protein was identified by Brew *et al.* (1968) as galactosyltransferase, while the R protein was shown by Brodbeck *et al.* (1967) to be α-LA. Interaction between the two components of lactase synthase (EC 2.4.1.22), α-LA, and galactosyltransferase, is essential for the catalysis of lactose biosynthesis. Because α-LA is involved in the last step in the biosynthesis of lactose it is critical for controlling lactation and section of milk (Brew *et al.*, 1968; Lo *et al.*, 1998). While α-LA has no catalytic function, it acts as a specific carrier protein in facilitating the action of galactosyl transferase, also known as $\beta_{1,4}$-galactosyltransferase (β_4Gal-T-1, EC 2.4.1.38). In the absence of α-LA, βGal-T-1 transfers galactose from UDP-galactose to *N*-acetyl-glucosamine. During the synthesis of lactose, manganese glucose, UDP-galactose, and galactosyl transferase combine with α-LA to form a dimer, which accepts millimolar concentrations of glucose, forming lactose under physiological conditions. α-LA appears to be the major regulator during lactogenesis by altering not only the sugar specificity of β-Gal-T-1 but also its sugar donor specificity (Ramakrishnan *et al.*, 2001, 2002). Thus, in the presence of α-LA, β-Gal-T-1 transfers galactose to glucose, which is its lactose synthase activity. Other possible regulators identified were D-glucose, UDP-galactose, calcium ions, and protein generation within the Golgi lumen, as well as the rate-limiting properties of the Golgi membrane.

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