9

Milk Salts: Technological Significance

J.A. Lucey and D.S. Horne

9.1. Introduction

Mammalian milk contains all the essential components to sustain the growth and development of the newborn suckling. Usually, this is taken to mean the protein, fat and carbohydrate, but it must also apply to the mineral components, the milk salts, including the citrates, phosphates and chlorides of H^+, K^+, Na^+, Mg^{2+} and Ca^{2+}, whether as ions in solution or as colloidal species complexed with the caseins. These minerals are essential for bone growth and development, for efficient cellular function or for maintaining osmolality in the wake of carbohydrate (lactose) synthesis. Like the other components, all these mineral species are there for a purpose and until weaning, milk may often be the only source of these essential elements.

There have been a number of reviews on the topic of milk salts (Allen, 1931; Pyne, 1962; Jenness and Patton, 1976; Walstra and Jenness, 1984; Holt, 1985, 1997; Fox and McSweeney, 1998; Gaucheron, 2005). In this chapter, the term salts will be used to represent substances that are, or can be, present in milk as low molecular weight ions. This group includes both inorganic and organic (e.g. citrate) substances. We can distinguish between the major salt constituents and trace elements and the latter will not be considered in this chapter. The approximate concentration of milk salts is shown in Table 9.1. The milks salts have a crucially important impact on many properties of milk, including the formation and stability of the casein micelles, acid–base buffering and various colligative properties, as well as its key biological role (i.e. providing nutrition for the newborn). In addition, these salts have a powerful

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Table 9.1. Approximate salt composition in milk (from various sources)

	Concentration			Concentration	
Cationic	mg l^{-1}	mmol kg^{-1}	Anionic	mg l^{-1}	mmol kg^{-1}
Calcium	1040–1280	26–32	Carbonate (including CO_2)	~200	~2
Magnesium	100–150	4–6	Chloride	780–1200	22–34
Potassium	1210–1680	31–43	Citrate	1320–2080	7–11
Sodium	350–600	17–28	Total phosphorus (PO_4) (all forms)	930–1000	30–32
			Inorganic phosphorus (as PO_4)	1800–2180	19–23
			Sulphate	~100	~1

influence on protein stability during processing (e.g. rennet coagulation, heat and alcohol stability), the texture of various types of milk protein gels, cheese texture and functionality and emulsion stability.

Milk is saturated with respect to calcium and phosphate ions, and these ions exist in a *dynamic equilibrium* with undissolved or colloidal forms (there is no true equilibrium for the Ca phosphates but some type of *pseudoequilibrium* that is influenced by several factors, including the presence of caseins). It has been recognized since Hammarsten (1879) that this insoluble Ca phosphate fraction is held in solution in the casein micelles (at that time the micelles were called the Ca caseinogenate). The partition of salts between the colloidal (micellar) and serum (soluble) phases is shown in Table 9.2 (the distribution between these two phases depends on the environmental conditions, including pH, temperature, concentration, etc.). In the serum phase, milk salts may be present as ion pairs (e.g. anions with cations). The Ca and Mg in milk are present at low concentrations as free ions, some as complexes with citrate and phosphate as well as significant amounts associated with

Table 9.2. Approximate distribution of salts between the colloidal and serum phases in milk (from various sources)

	Colloidal (micellar) (%)	Serum (soluble) (%)
Calcium	69	31
Chloride	5	95
Citrate	14	86
Inorganic phosphate	53	47
Magnesium	47	53
Potassium	6	94
Sodium	5	95

Table 9.3. Calculated values for the major forms of calcium and magnesium in milk (mainly adapted from Neville, 2005)

Binding species	Concentration (mmol l^{-1})
Calcium	
$[Ca^{2+}]$	2.0
$[CaCit^{-}]$	6.9
$[CaPO_4^{-}]$	0.6
Casein	19.4
α-lactalbumin	0.5
Magnesium	
$[Mg^{2+}]$	0.8
$[MgCit^{-}]$	2.0
$[MgPO_4^{-}]$	0.3
Casein	1.9

casein micelles (Table 9.3). Both Mg and citrate are present in the colloidal phase, which is remarkable since their concentrations (or activities) are not in excess of solubility (Walstra and Jenness, 1984). The Ca and phosphate contents vary in proportion to the casein content of milk since much of the Ca and phosphate are associated with the casein micelles.

9.2. Methods of Analysis

Ashing (heating in a muffle furnace at ~550°C) of milk is an approximate method of quantifying the inorganic elements (0.7–0.8% in normal milk but values >1.3% can be found in colostrum). However, organic salts are lost during ashing. Some carbonates are lost during ashing (as CO_2) and some carbonates are formed from organic compounds. Phosphates from lipids (i.e. phospholipids) also appear in the ash. The sulphur of proteins is oxidized during incineration and appears as sulphate. Oxidation also results in the formation of metal oxides. Ashing is routinely used as a pre-treatment (by oxidizing organic matter) step for elemental analysis as the ash can be dissolved with acid and used for quantification of Ca, Fe, etc. by atomic absorption spectroscopy. The various techniques used for the analysis of milk salts were described by Fox and McSweeney (1998). Partition of salts between the colloidal and dissolved forms can be achieved by dialysis, ultrafiltration and the preparation of rennet whey (Davies and White, 1960; de la Fuente *et al.*, 1996), although some adjustments (e.g. to account for excluded volume effects) must be made with these techniques to calculate the serum concentration.

9.3. Secretion of Milk Salts

The biosynthesis of components in milk and milk secretion have been reviewed many times (e.g. Blackwood and Stirling, 1932; Petersen, 1944; Linzell and Peaker, 1971; Larson, 1985; McManaman and Neville, 2003). The cytoplasm of lactating alveolar cells is filled with numerous mitochondria and an extensive rough endoplasmic reticulum network. In addition, there is a well-developed Golgi apparatus, and secretory vesicles containing casein micelles are present in the apical region of the cell. Epithelial cells are connected to each other through an apical junctional complex composed of adherens and tight junctional elements that function to inhibit direct paracellular exchange of substances between vascular and milk compartments during lactation (McManaman and Neville, 2003).

The secretion of milk salts has been reviewed by Holt (1981, 1985) and Neville (2005). Lactating mammals must supply large amounts of Ca to the mammary gland where it is transported across mammary epithelial cells and into milk. Calcium is pumped from the cytoplasm into the Golgi compartment and enters milk via exocytosis of secretory vesicles from the Golgi compartment with a membrane-associated Ca ATPase mediating the transport (Bingham *et al.*, 1993). Circulating Ca concentration must remain relatively constant, i.e. Ca homeostasis; a number of diseases/conditions occur when this is not the case. Such stability relies on cooperation between several organs, principally the parathyroid glands, the kidneys, the skeleton and the gut. Several important entities are involved in the feedback loop that regulates Ca fluxes to the mammary gland. These control features include an extracellular Ca-sensing receptor (CaR) and parathyroid hormone-related protein (PTHrP) (VanHouten, 2005). Very high concentrations of Ca are transferred from the cytoplasm although the cytoplasmic Ca concentration remains relatively constant (in the μM range). This demand for Ca is associated with transient loss of bone mass (in humans), triggered, in part, by the secretion of PTHrP from the mammary gland into the circulation (Ardeshirpour *et al.*, 2006). The CaR is a G-protein-coupled receptor that signals in response to extracellular Ca^{2+} (Ardeshirpour *et al.*, 2006). It is responsible for coordinating Ca homeostasis by regulating parathyroid hormone secretion and by regulating Ca handling in the renal tubules. Calcium activates basolateral CaRs to stimulate its own transport into milk (VanHouten *et al.*, 2004). The intracellular Na and K concentrations are established by a Na/K-activated ATPase on the basolateral surface of the secretory cell, and there is a dynamic electrochemical equilibrium of these ions across the apical membrane (Holt, 1985).

It has been known for a long time that milk is in osmotic equilibrium with blood, i.e. milk is isotonic with blood (van der Laan, 1915). Taylor and

Husband (1922) were probably the first to suggest that the quantity of lactose produced by the mammary gland controls the daily volume of the milk. Koestler (1920) used the ratio of lactose and chloride as a method to indicate normal and mastitic (abnormal) milk. The Koestler number is given by (100 × chlorine %)/lactose %. Normal milk has a Koestler number less than 3 while mastitic milk is considerably higher (e.g. 15). One of the first studies of the possible mechanisms involved in the secretion of Ca and phosphate in milk was reported by Wright (1928).

The large amounts of phosphate required by the suckling for normal growth and development are also supplied through the milk in at least three chemical forms, namely free inorganic orthophosphate in solution, colloidal phosphate associated with Ca in micellar Ca phosphate and the ester phosphate of the caseins. The major pathway for phosphate secretion into milk is believed to be the Golgi vesicle route by a Na^+-P_i co-transport mechanism (Shennan and Peaker, 2000). Holt (1985) described another possible mechanism by which phosphate is generated in the Golgi lumen by hydrolysis of UDP during lactose synthesis (Kuhn and White, 1977). This uridine nucleotide cycle involves UDP-galactose and glucose. Within the vesicle, these precursors form UDP and lactose. The UDP cannot cross the vesicle membrane unless hydrolysed to UMP and inorganic phosphate, both of which can re-enter the cytosol, avoiding product inhibition of lactose synthetase. However, the widely varying concentrations of lactose found in milks of different mammalian groups suggest that other routes for P_i transport across the Golgi membrane may exist conjointly with the UDP hydrolysis mechanism. These may involve the ATP-driven Ca^{2+} pump discussed above, driving the accumulation of Ca^{2+} and actively participating in the phosphorylation of casein. Here, P_i is a by-product of the hydrolysis of ADP produced in that phosphorylation reaction (Shennan and Peaker, 2000).

Citrate concentration in milk varies widely throughout lactation (Banks *et al*., 1984). In general, citrate levels are higher during the grazing season (Holt and Muir, 1979) and during early lactation (Braunschweig and Puhan, 1999; Garnsworthy *et al*., 2006). In studies on the goat, Linzell *et al*. (1976) found that the mammary epithelium is impermeable to citrate in both directions, suggesting that citrate is synthesized within the secretory cells and released into milk after exocytosis of Golgi vesicles. Citrate has an indirect role in fat synthesis by providing reducing equivalents in the form of NADPH, which are required for de novo synthesis of fatty acids (Faulkner and Peaker, 1982). Citrate is in equilibrium with iso-citrate which is converted to α-ketoglutarate in the production of NADPH. Thus, increased de novo synthesis of fatty acids is predicted to lead to a decrease in citrate concentration. Such a correlation was found in the studies of Banks *et al*. (1984) who

used fat supplements to reduce de novo synthesis of fatty acids in the mammary gland and induce increases in milk citrate concentration and is confirmed in the more recent lactational studies of Garnsworthy *et al.* (2006). The latter authors found a significant correlation between milk citrate and the amounts of acetate required for chain elongation in de novo fatty acid synthesis. Any change in the citrate concentration of milk would therefore directly influence the Ca^{2+} concentration (as citrate readily binds Ca^{2+}), which could influence the functionality of milk, e.g. its rennet coagulation time. This type of mechanism could account for at least some of the observed seasonal or diet-related changes in milk functionality.

It has recently been proposed that casein-derived phosphopeptides disrupt tight junction integrity and precipitously cause milk secretion to dry up, i.e. they may help trigger the involution process (Shamay *et al.*, 2002). It is known that plasmin activity increases near the end of lactation (Politis *et al.*, 1989) and it is possible that some phosphopeptides are produced by this mechanism.

9.4. Factors Influencing the Milk Salts Equilibria

There are numerous dynamic equilibria between the salts in milk, and changes in many environmental conditions influence these equilibria. Some of these changes occur relatively quickly but those involving colloidal Ca phosphate (CCP) can be slow. Mastitic infections of the udder result in a decrease in the concentrations of Ca^{2+} and K^+ in milk but an increase in the concentrations of Na^+ and Cl^- (due to leakage of these ions into milk from blood where their concentrations are much higher than in milk). It should be noted that during milking and processing most CO_2 is lost. The impact of various processing techniques on the milk salts equilibria has been reviewed (Holt, 1985; de la Fuente, 1998; Gaucheron, 2005).

9.4.1. Temperature

Milk as secreted by the cows probably contains about 20 mg of CO_2 per 100 ml (Jenness and Patton, 1976). This gas is lost rapidly and heating and agitation accelerate this loss. The pH of milk decreases as its temperature increases, although few measurements of pH have or can be made at very high temperatures.

The solubility of Ca phosphates decreases at high temperature and during heating heat-induced CCP is formed, which re-solubilizes when milk is cooled subsequently. Jenness and Patton (1976) roughly gave that reaction as

$$3Ca^{2+} + 2HPO_4^{2-} \rightarrow Ca_3(PO_4)_2(\text{precipitate}) + 2H^+$$

The release of H^+ contributes to the decrease in milk pH observed on heating (with extreme heating there is also the production of organic acids, principally formic from lactose) (Dalgleish, 1989). This heat-induced CCP appears to associate with the existing CCP in casein micelles, possibly by increasing the size of the nanoclusters (Holt, 1995). The original equilibrium is mostly restored after cooling but there is some hysteresis. Cooling and holding milk at low temperatures result in an increase in the solubility of Ca phosphate and thus a decrease in the concentration of CCP. The Ca^{2+} activity is also restored if sufficient time is allowed for equilibration (Geerts *et al.*, 1983; Augustin and Clarke, 1991). At temperatures $\geq 40^\circ C$, artificial milk serum buffers (or ultrafiltrate) are prone to precipitation. Caseins are effective stabilizers of CCP and usually prevent precipitation of these salts in milk. The absence of casein from these buffers alters the behaviour of salts during heating and irreversible precipitation of Ca phosphate occurs (Holt, 1995). The deposits found on the surfaces of ultra-high-temperature heat exchangers are rich in Ca phosphate. There are indications that very severe heat treatments (e.g. 120°C for 15 min) cause a change in the nature of CCP, as indicated by an altered acid–base buffering profile (Lucey *et al.*, 1993a), e.g. to form hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; Visser *et al.*, 1986); under these severe heating conditions caseins are unable to prevent the precipitation of Ca phosphate (Holt, 1995).

Holding milk at low temperatures causes dissociation of some caseins, especially β-casein (<20% of total β-casein) (Downey and Murphy, 1970; Creamer *et al.,* 1977) and some dissolution of CCP (Qvist, 1979; Ali *et al.*, 1980). Most of these changes are reversed readily by mild heating, e.g. pasteurization (Qvist, 1979).

Freezing of milk is sometimes practiced where milk production is seasonal, e.g. goat and ewe milk for cheese making (Wendorff, 2001). Freezing and thawing for a sufficient time result in the reversal of most of the changes in the salt equilibrium that may have been caused during freezing. Long-term storage (after several months) of milk at $\leq -15^\circ C$ can result in protein precipitation (see Chapter 1). Ovine and caprine milk stored frozen for a few months had similar levels of soluble Ca, Mg and P after thawing as in the unfrozen milk (de la Fuente *et al.*, 1997).

9.4.2. pH

Acidification solubilizes CCP which is an integral part of casein micelles. The extent of solubilization increases markedly below pH 5.6 and is complete at approximately pH 5.0 (Pyne and McGann, 1960; Brule *et al.*,

1974; Pierre *et al.,* 1983; van Hooydonk *et al*., 1986; Dalgleish and Law, 1989; Mariette *et al*., 1993). The pH at which CCP is completely solubilized presumably varies with the conditions (e.g. rate and temperature) of acidification. At pH values <5 milk is unsaturated with respect to most types of calcium phosphate (Lyster, 1979). At high pH values (<6), concentrated milk products (e.g. processed cheeses) have an increased likelihood of precipitation of some types of Ca phosphate. Increasing the pH of milk results in the formation of additional CCP. McGann and Pyne (1960) described a method for increasing the CCP content of milk (by up to 200%). Milk pH was increased by the addition of NaOH at about 0°C followed by exhaustive dialysis against a large excess of the original milk.

Lucey *et al*. (1996) studied the impact of (cold) acidification and neutralization of milk on the properties of casein micelles. Acidification of milk to pH 5.0 or 4.6, followed by neutralization to pH 6.6, resulted in a reduction in the buffering maximum of milk at pH ~5.1; this buffering peak is caused by the solubilization of CCP. The reduced buffering in reformed milk suggests that little reformation of CCP occurs on neutralization; reformed milks also had an elevated Ca^{2+} activity. Acidification of milk to pH > 5.5, followed by neutralization to pH 6.6, hardly reduced buffering (at pH ~5.1), suggesting that either little CCP dissolved on acidification in that pH range or reformation of CCP occurred on neutralization. Canabady-Rochelle *et al*. (2007) also reported that milk had a higher soluble Ca level after acidification and neutralization.

Gevaudan *et al*. (1996) used high-pressure CO_2 to acidify milk reversibly (pH was restored to the original value after depressurization). Acidification to pH ~5 with high-pressure CO_2 resulted in a reduction in the buffering peak at pH ~5.1 but this peak increased during chilled storage of this milk (Raouche *et al*., 2007).

Heat treatment has little impact on the pH-dependent release of Ca and phosphate from micelles during acidification (Law, 1996; Singh *et al*., 1996).

9.4.3. Concentration of Milk

Concentrating milk by evaporation results in a decrease in milk pH, e.g. a decrease of ~0.3 and 0.5 pH units for 2:1 and 3:1 concentrations, respectively (Walstra and Jenness, 1984). The [Ca^{2+}] increases with concentration but less than the concentration factor (Walstra and Jenness, 1984). Presumably, the smaller increase in Ca^{2+} is at least partly due to the formation of additional CCP (even though the pH decreases in evaporated milk). Membrane filtration of milk using either ultrafiltration or microfiltration results in retentates where CCP is a greater proportion of the total Ca content as some soluble Ca is lost in the permeate during processing (Lelievre and Lawrence

1988; Srilaorkul *et al*., 1989; Solanki and Rizvi, 2001). In the production of highly concentrated (casein content $\geq$70%) milk protein powders (e.g. milk protein concentrates, MPC), extensive diafiltration or washing is required to reduce the lactose content. This extensive washing removes most soluble Ca, and partly reduces the CCP content in micelles and causes some casein dissociation. It is well known that extensive dialysis of casein micelles against water causes dissociation of caseins due to the loss of CCP.

9.4.4. Ca Sequestrants or Chelating Agents

Sequestrants (e.g. citrates and phosphates) combine with polyvalent metal ions (e.g. Ca^{2+} or Mg^{2+}) to form a soluble metal complex. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), are complexes in which the metal ion is bound to two or more atoms in the chelating agent, usually in the form of a ring-type structure. The addition of sequestrants or chelating agents to milk disrupts casein micelles by reducing the [Ca^{2+}] and CCP content (Munyua and Larsson-Raznikiewicz, 1980; Visser *et al*., 1986; Udabage *et al*., 2000), which causes casein micelle dissociation (Morr, 1967; Gaucheron, 2005). Several studies have reported that some of the CCP cross-links can be removed from micelles without causing a lot of protein dissociation; higher levels of Ca removal caused micellar disintegration (Lin *et al*., 1972; Griffin *et al*., 1988). Udabage *et al*. (2000) found that adding high concentrations of sequestrants or chelating agents resulted in a significant reduction in micelle diameter and a very large reduction in particle light scattering.

Removal of Ca from milk using an ion exchange resin resulted in an increase in pH, a reduction in Ca^{2+}, an increase in ethanol stability and an increase in the rennet coagulation time (Lin *et al*., 2006).

When comparing the various types of phosphates, the orthophosphates are relatively poor at complexing Ca. Comparing the ability to complex Ca, phosphates and citrates can be ranked in the following order: long-chain phosphates > tripolyphosphate > pyrophosphate > citrate > orthophosphate (Van Wazer and Callis, 1958). Figure 9.1 shows a comparison of the [Ca^{2+}] remaining in solution in equilibrium with a 0.01 M solution of a number of sequestering agents (Van Wazer and Callis, 1958). This figure demonstrates the relative complexing abilities of the orthophosphates (weak, more free Ca left in solution) with long-chain polyphosphates (strong, little free Ca left in solution).

In well-defined systems, the relative efficiency of sequestrants can be compared by looking at the stability constants (formation constant, equilibrium constant) for a given metal (Furia, 1972). In general terms the

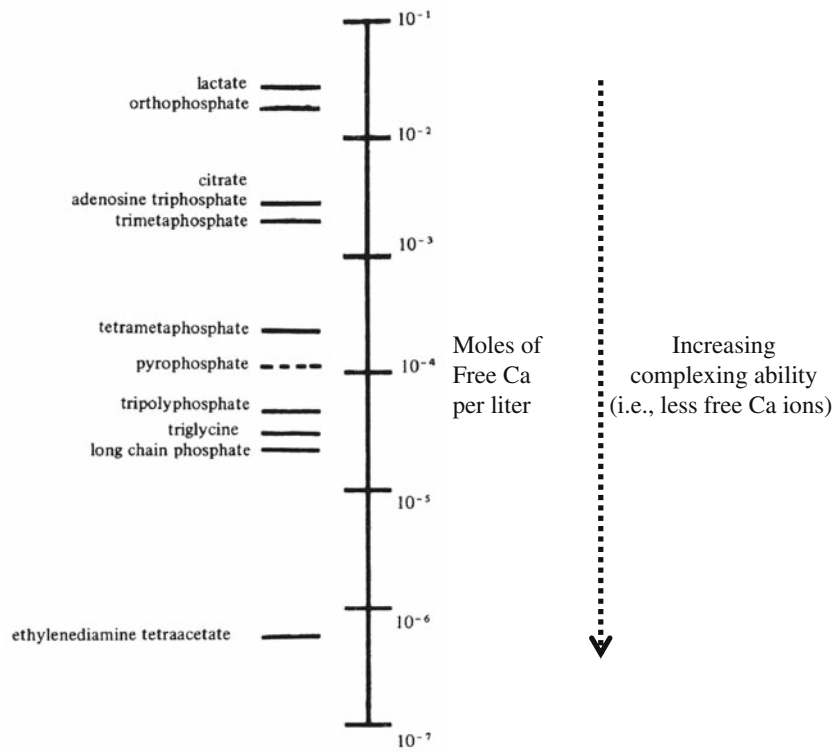

Figure 9.1. The free calcium concentration (i.e. not chelated or sequestered) for various types of complexing agents are estimated for the dissociation of a 0.01 M solution of the 1:1 Ca complex. Complexing agents that are lower on the scale (e.g. EDTA) are stronger chelators for calcium (adapted from Van Wazer and Callis, 1958).

stability constant of a metal (e.g. Ca^{2+}) complex can be calculated as follows (Furia, 1972):

$$K = \frac{[ML]}{[M]\,[L]}$$

where M is the metal ion, L is the ligand (sequestrant, chelating agent) and ML is the metal complex.

The (log K) stability constants for Ca-chelates with citrate, pyrophosphate and EDTA are 3.5, 5.0 and 10.7, respectively (Furia, 1972). Higher values indicate a stronger tendency to form a complex.

The highly charged anionic nature of polyphosphates causes them to be attracted to, and to orient themselves along, the charged sites of other long-chain polyelectrolytes such as proteins (Van Wazer and Callis, 1958). This should increase the charge repulsion between caseins at pH values above their isoelectric point (as in most dairy processing situations). At pH values below the isoelectric point, polyphosphates can induce protein precipitation by cation–anion interactions (Van Wazer and Callis, 1958). Casein has been reported to precipitate or aggregate in the presence of phosphates (Fox *et al*., 1965). Some types of phosphates can crosslink caseins, e.g. pyrophosphates, and they can even induce casein gelation (Mizuno and Lucey, 2005, 2007). Phosphate salts have also been used to cause heat-induced aggregation of caseins (Panouillé *et al*., 2003).

9.4.5. High Pressure

High hydrostatic pressure (HHP) reduces the light scattering of milk due to the disruption of casein micelles (Schmidt and Buchheim, 1970). HHP influences various properties of milk, including a reduction in the size of casein micelles, denaturation of β-lactoglobulin and a reduction in CCP content (see reviews by Huppertz *et al*., 2002; López-Fandiño, 2006). HHP treatment influences the functional properties of proteins through the disruption of hydrogen bonds and hydrophobic interactions and the separation of ion pairs. The impact on the properties of casein depends not only on the pressure applied but also on factors such as the application time, pH and temperature. It is well known that very high hydrostatic pressure $\geq$300 MPa causes the disintegration of the casein micelles as observed by a reduction in particle size (Needs *et al*., 2000; Garca-Risco *et al*., 2003). Micelle size is hardly unaffected, or is slightly increased, by pressures up to 250 MPa (Needs *et al*., 2000; Huppertz *et al*., 2004). Concomitantly with these size changes there is dissociation or aggregation (when there is an increase in size) of caseins. Huppertz and de Kruif (2006) proposed that the unfavourable exposure of hydrophobic surfaces at a pressure >200 MPa leads to the formation of larger casein particles from fragments of disrupted casein micelles during prolonged HHP treatment. The interactions responsible for this re-association were likely to include van der Waals or hydrophobic interactions.

HHP treatment solubilizes some of the CCP of raw (Schrader *et al.,* 1997; López-Fandiño *et al*., 1998) and heat-treated milk (Gaucheron *et al*., 1997; Schrader *et al*., 1997). Some or nearly all of the CCP is restored during subsequent storage of HHP-treated milk (Gaucheron *et al*., 1997: Schrader *et al*., 1997; Huppertz *et al*., 2006). Similar trends have been observed for milk of various species although the magnitude of the changes in the state of the CCP varied (López-Fandiño *et al*., 1998; Huppertz *et al*., 2006). Some studies

have found hardly any change in the concentration of soluble Ca after HHP treatment (Law *et al*., 1998). It is presumed that during HHP the solubilization of some of the CCP helps to cause casein micelle disintegration by disrupting one of the key crosslinking agents within micelles. Although pressure release helps to reverse the increase in soluble Ca during pressurization, the original micelle structure is not reformed (Law *et al*., 1998).

High-pressure CO_2 has been used as a recyclable acid for the isoelectric precipitation of casein (Hofland *et al*., 1999). After the pressure is released precipitation of Ca phosphate occurs (as the pH is also restored to the original value).

9.5. Impact of Milk Salts on the Buffering Properties of Milk and Dairy Products

The buffering properties of dairy products have been reviewed by Singh *et al*. (1997) and Salaün *et al*. (2005). The affinity of acids and bases for H^+ may be expressed in terms of titration curves and dissociation constants. An acid–base titration curve is a plot of pH versus the amount of acid or base neutralized in the titration. The buffering value (index) at any pH may be determined graphically from the slope of the tangent to the titration curve at that pH. If the added alkali or acid is *dB* and the resulting change in pH is *dpH,* then the average buffering value, i.e. the amount of acid or base required to cause a predetermined change in pH (dpH), is the differential ratio, *dB*/*dpH* (Van Slyke, 1922), where

$$\frac{dB}{dpH} = \frac{(\text{ml of acid or base added}) \times (\text{normality of acid or base})}{(\text{average volume of sample}) \times (\text{pH change produced})}$$

Apart from casein, the principal buffering components in milk are soluble phosphate, CCP, citrate and bicarbonate. Srilaorkul *et al*. (1989) estimated that the contribution of casein, whey proteins and milk salts to the buffering of skim milk was 36.0, 5.4 and 58.6%, respectively. Lucey *et al*. (1993b) reported that in the pH range 6.7–4.0, soluble salts and whey proteins (i.e. the substances in rennet whey), CCP and casein contributed approximately 47, 21 and 32%, respectively, to buffering in milk.

When milk is acidified (Figure 9.2a), maximum buffering occurs at approximately pH 5.1 but when acidified milk is back-titrated with base, there is low buffering at pH 5.1 and maximum buffering occurs at pH ~6.3. The maximum in the buffering curve at pH ~5.1 is due to the solubilization of CCP, which results in the formation of phosphate ions that combine with H^+

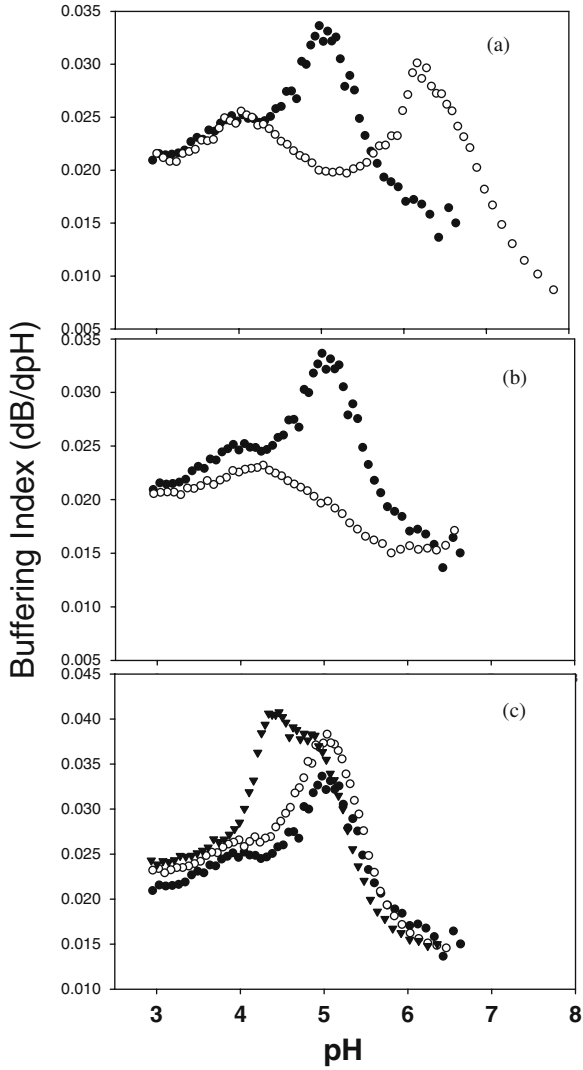

Figure 9.2. Acid–base buffering curves of (**a**) milk titrated from its initial pH to pH 3.0 with 0.5 N HCl (•) and back-titrated to pH 8.0 with 0.5 N NaOH (○); (**b**) milk (•) and colloidal calcium phosphate-free milk (○) titrated from the initial pH to pH 3.0 with 0.5 N HCl; (c) titration of unheated milk (•), milk heated at 100°C for 10 min (○), milk heated at 120°C for 15 min from the initial pH to pH 3.0 with 0.5 N HCl (▼) (adapted from Lucey *et al.*, 1993a,b).

(to form HPO_4^{2-} and $H_2PO_4^-$), resulting in pH buffering. The removal of the CCP from milk, as in CCP-free milk made by the method of Pyne and McGann (1960), results in the absence of the buffering peak at pH 5.1 during acid titration (Figure 9.2b). When the acidified milk sample is back-titrated with base, buffering is low at pH 5.1, because CCP is already solubilized, but maximum buffering occurs at pH 6.3, due to the formation of Ca phosphate (precipitation) with the release of H^+ (from HPO_4^{2-} and $H_2PO_4^-$) which can combine with OH^-.

High heat treatments cause an increase in CCP due to the formation of heat-induced CCP (Figure 9.2c). Some of the heat-induced CCP solubilizes on cooling (depending on the equilibration time allowed) but there is a substantial shift in the type of buffering curve observed during the acidification of very severely heated milk (e.g. 120°C for 15 min) (Figure 9.2c).

A strong buffering effect in the pH range 6–7 arises from the formation of Ca phosphate as can be seen in the titration of phosphoric acid in the presence of Ca (Figure 9.3). This buffering effect due to precipitation of Ca phosphate has been reported by many investigators, e.g. Visser (1962). Due to the precipitation of Ca phosphate around pH 6, the titration behaviour of phosphoric acid in the presence of Ca is completely different from that when this titration is performed in the absence of Ca (Figure 9.3). In milk, both Ca and phosphate are present which suggests that this behaviour would occur in dairy products. As is shown in Figure 9.3, the onset of precipitation of Ca phosphate results in the release of H^+. We can speculate that this release of H^+ could also occur during the formation of CCP in the mammary gland and may contribute to the lower pH of milk compared to that of blood.

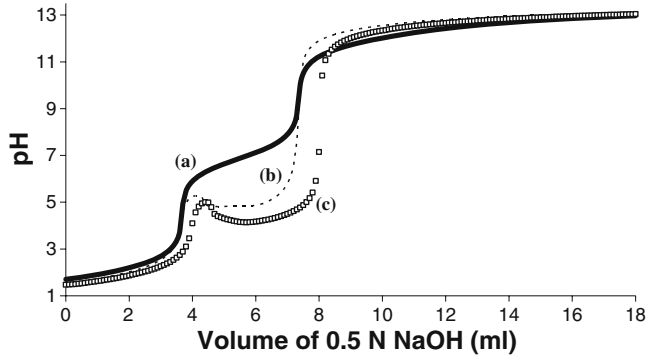

Figure 9.3. Potentiometric titrations of 400 mg phosphoric acid with 0.5 N NaOH in the presence of (**a**) no calcium, (**b**) 325 mg $CaCl_2$, (**c**) 650 mg $CaCl_2$; the method reported by Visser (1962) was used for these titrations (Salim and Lucey, unpublished data).

Acid–base buffering analysis is now widely used to indicate changes in the amount and type of CCP in milk as influenced by various technological treatments (e.g. Mizuno and Lucey, 2005).

9.6. Interactions Between Milk Salts and Casein

9.6.1. Introduction

Caseins constitute approximately 80% of the protein in bovine milk, with four main types (α_{s1}-, α_{s2}-, β- and κ-caseins) (casein fragments can be produced as a result of proteolysis). Caseins are found in combination with appreciable quantities of micellar or CCP, sometimes called CCP nanoclusters, in the form of aggregates called casein micelles (Holt, 1992). Casein plays a critical role in making milk super-saturated with Ca phosphate. As a packaging system, the micelles convert the milk into a free-flowing, low-viscosity fluid and provide the means to transport the high levels of Ca and phosphate at concentrations which would normally precipitate in the mammary gland in the absence of the caseins. The CCP is completely soluble at pH values < 5 (Pyne and McGann, 1960; Lyster, 1979) and the released Ca and phosphate are then available for absorption by the digestive system.

The caseins are a family of phosphoproteins found in the milk of all mammals. They are members of the group of Ca-phosphate-sequestering proteins which include dentine, bone matrix proteins and salivary proteins, amongst others (Kawasaki and Weiss, 2003). Phosphorylation is a post-translational modification of the caseins and it occurs at the serine groups, or rarely threonine, following a recognized template sequence Ser-X-Y, where X is any amino acid and Y = Glu, Ser-P or Asp. Due to the placing of the serine residues along the molecular sequences of α_{s1}-, α_{s2}- and β-caseins, most of the phosphorylated residues are found in clusters. Thus, four of the five phosphorylated serine residues in bovine β-casein are found in the sequence of residues 15–19, with the fifth at position 35. Four of the eight serines in bovine α_{s1}-casein are located in the sequence 64–68 with two more downstream at positions 46 and 48 and one upstream at position 75. Bovine α_{s2}-casein can have a variable level of phosphorylation from 10 to 13 moles P per mole of protein. The most abundant of these, α_{S2}-casein-11P, has three groupings of phosphorylated residues, one cluster of three from residues 8 to 10, four SerP spread as a group of three from 56 to 58 with the fourth member at 61, with the third cluster of two at positions 129 and 131. The remaining two single Ser-P residues of the total eleven are located at positions 16 and 135 (Horne, 2002). κ-Casein is unique amongst

the caseins in the absence of phosphoseryl clusters. Most molecules of κ-casein contain only one phosphoserine residue, rarely two or three, and all singlets located in the hydrophilic C-terminal region. The caseins are therefore sensitive to coagulation or precipitation by Ca. Horne and Dalgleish (1980) demonstrated that the logarithm of this critical coagulation time is a linear function of Q^2, where Q is the net negative charge on the protein, taking into account the binding of Ca to the casein. This linear correlation was also maintained when protein charge was changed following chemical modification of charged residues along the protein chain (Horne, 1979, 1983; Horne and Moir, 1984).

9.6.2. Casein Micelle Formation

The caseins are sensitive to precipitation due to the presence of approximately 30 mM Ca in milk. However, a key biological purpose of milk is to provide high concentrations of the essential Ca and phosphate required for the growth of the newborn mammal. So how are these two conflicting factors resolved? The solution involves casein micelle formation, the formation of an insoluble CCP phase within the micelles and the requirement for one of the caseins (usually κ-casein) to be insensitive to Ca and provide stability against Ca-induced precipitation to the other caseins. The details of how this occurs have been the subject of much debate and intensive study. For a discussion of the various casein micelle models, the reader is referred to various reviews (Farrell, 1973; Slattery, 1976; Rollema, 1992; De Kruif and Holt, 2003; Farrell *et al*., 2006; Qi, 2007). We will focus our explanations on the dual-binding approach for micelle formation as described by Horne (1998, 2002, 2006, 2008).

In the dual-binding model (Figure 9.4), micellar assembly and growth take place by a polymerization process involving two distinct forms of bonding/interactions, namely association through clustering of hydrophobic regions/patches of the caseins and second, linking of several phosphopeptides into the Ca phosphate nanoclusters. Central to the model is the concept that bond formation is facilitated, and hence micellar integrity and stability are maintained, by a local excess of hydrophobic attraction over electrostatic repulsion (otherwise if the repulsive interactions were too large, little association of casein would occur and micelle formation would not be observed in milk). It should be noted that there are quite different ranges for these interaction components. Compared to hydrophobic interactions, electrostatic repulsion is a long-range force. Clustering of charged groups in specific regions of the protein molecule means that electric dipole moments may be

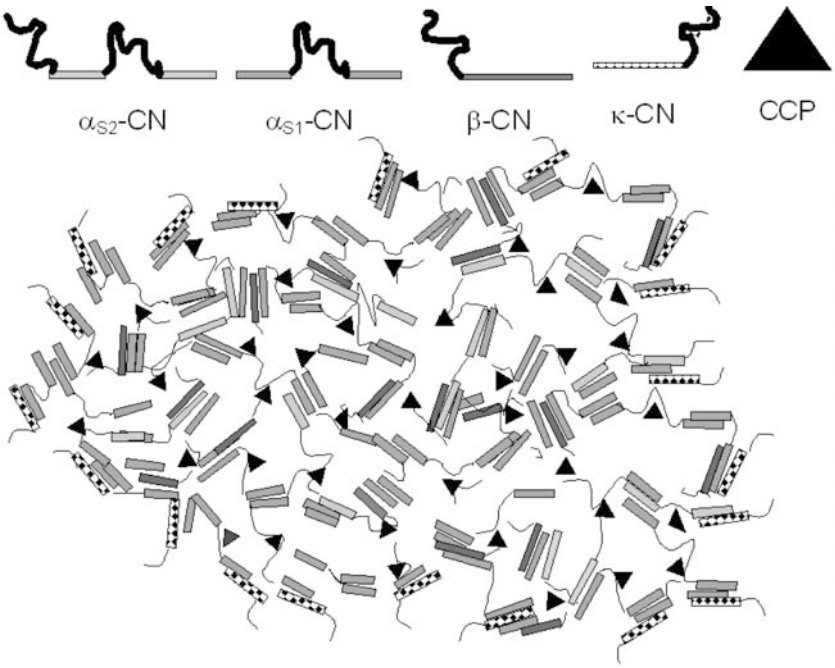

Figure 9.4. Dual-binding model for the casein micelle. CN is casein and CCP is colloidal calcium phosphate (Horne, 1998).

large, so that their effects on interparticle interactions may be rather strong (Piazza, 2004).

Each casein molecule effectively functions as a block copolymer, with the hydrophobic region(s) offering the opportunity for a multitude of individual, weak, hydrophobic interactions (driven by the thermodynamically favourable exclusion of water by this type of association). The hydrophilic regions of the casein molecules contain the phosphoserine cluster (or clusters), with the exception of κ-casein which has no such cluster, each offering multiple functionality for crosslinking. α_{s1}-Casein can polymerize (self-associate) through the hydrophobic blocks, forming a worm-like chain. Further growth is limited by the strong electrostatic repulsion of the hydrophilic regions, but in the casein micelle the negative charges of the phosphoserine clusters are neutralized by intercalating their phosphate groups into a facet of the Ca phosphate nanoclusters. This has two very important implications for the micelle. First, by removal of a major electrostatic repulsion component, it increases the propensity for hydrophobic bonding upstream

and downstream of the nanocluster link. It effectively permits and strengthens those bonds. Second, it allows for multiple protein binding to each nanocluster (on different facets), allowing a network to be built up. β-Casein, with only two blocks, a hydrophilic region containing its phosphoserine cluster and the hydrophobic C-terminal tail, can form polymer links into the network through both, allowing further chain extension through both. α_{s2}-Casein is envisaged in this model as having two of each block, two (possibly three, see below) phosphoserine clusters and two hydrophobic regions. It is only a small fraction of the total bovine casein but, by being able to sustain growth through all its blocks, it is likely to be bound tightly into the network. α_s-Caseins cannot be essential to micelle formation as human milk contains only trace amounts ($\sim$0.06% of total protein) of α_s-caseins (Lönnerdal, 2004) and yet micelles are formed. κ-Casein is the most important of the caseins in the dual-binding model of micellar assembly and structure. It can link into the growing chains through its hydrophobic N-terminal block but its C-terminal block is hydrophilic and cannot sustain growth by linking hydrophobically to another casein molecule. Nor does κ-casein possess a phosphoserine cluster and therefore it cannot extend the polymer cluster through a nanocluster link. Thus, chain and network growth are terminated wherever κ-casein joins the chain.

This polymerization process leaves the network with an outer layer dominated by κ-casein although other caseins are also present at, or close to, the surface (Dalgleish, 1998). Srinivasan and Lucey (2002) studied the impact of plasmin on the rennet coagulation of skim milk. They found that even partial hydrolysis of β- and α_s-caseins accelerated the rennet coagulation of milk. Plasmin hardly degrades κ-casein. Srinivasan and Lucey (2002) hypothesized that plasmin could have degraded β-casein "hairs" present on the surface of micelles and that this could have reduced the repulsive barrier to aggregation of rennet-altered micelles such that aggregation could occur at a lower degree of κ-casein hydrolysis.

Other evidence that some β-casein may be on or close to the micelle surface is that a considerable proportion (up to about 20%) of β-casein can dissociate from the micelle at low temperatures (this also occurs in ovine micelles but to a much lesser extent in porcine milk due to its high CCP content; Umeda, 2005; Umeda and Aoki, 2005; Umeda *et al.,* 2005). Some CCP also dissolves at low temperatures and that occurrence might also weaken the interactions between β-casein molecules and the rest of the micelle. How can a considerable proportion of β-casein dissociate at low temperatures while little κ-casein dissociates even though κ-casein is mainly on the surface? It is possible that κ-casein becomes polymerized by S–S bridging between κ-casein molecules (Farrell *et al*., 1996) after it has terminated the growth of the casein chains. If κ-casein polymers are formed

(in vivo), it is likely that these polymers would have greater attachment/linkage to the rest of the micelle structure, making them more difficult to remove. Also, the attractive balance in κ-casein is not very sensitive to changes in phosphoserine involvement in CCP nanoclusters (so the loss of some CCP crosslinks at low temperatures does not have a major impact on its dissociation from the micelle) as they do not interact through CCP crosslinks. In contrast, for β-casein, if some of the CCP crosslinks are dissolved at low temperatures then the exposed negative charge on the phosphoserine residues would make the binding of β-casein to other casein molecules unfavourable. This type of process could allow some of the β-casein to dissociate as the temperature is lowered. It is likely that the β-casein that dissociates is closer to the micelle surface or if not, then the β-casein freed by this process would have some potential chances to re-attach/associate with other caseins as it diffuses through the inner micelle network out to the bulk solution.

κ-Casein-deficient mice, produced by genetic modification, were unable to lactate because of destabilization of the micelles in the lumina of the mammary gland (Shekar *et al.*, 2006). The milk of various species appears to have a κ-casein or Ca-stabilizing casein (i.e. a casein that does not have a phosphate cluster), whereas some milks contain little or no α_s-caseins (human milk) and various ratios of α_s- to β-caseins.

9.6.3. Nature of Colloidal Calcium Phosphate

The nature of CCP or micellar Ca phosphate (as it is sometimes called) has been the subject of intense study and debate over the years. There have been several reviews of the nature of CCP (Pyne, 1934; McGann and Pyne, 1960; Schmidt, 1980; van Dijk, 1990; Holt, 1992, 1995; De Kruif and Holt, 2003). Schmidt (1980, 1982) considered the CCP to be a ubiquitous coating or "cement" that bonded many casein molecules together. McGann *et al.* (1983a,b) reported that the CCP depositions in milk systems consist of spherical granules (other later names for these granules include nanoclusters) 2–3 nm in diameter. Such a large entity is incompatible with the small type of CCP structures proposed by van Dijk (1990) or Schmidt (1980).

For many years, CCP was believed to be a basic Ca phosphate salt (e.g. Pyne and McGann, 1960). Pyne and McGann (1960) and McGann *et al.* (1983a) reported that in CCP, the Ca/P_i ratio is >1.5, which would make it some type of basic salt, like apatite or tricalcium phosphate (e.g. $Ca_3(PO_4)_2$). Citrate and magnesium are also associated with the CCP phase. Various other studies have suggested that CCP is a more acidic phase, such as some brushite-type structure ($CaHPO_4$) (e.g. Holt *et al.*, 1982). Various groups have considered CCP to be amorphous, i.e. lacking a crystalline structure (Pyne and

McGann, 1960; Knoop *et al.*, 1979; McGann *et al.*, 1983b; Lyster *et al.*, 1984), although we believe that it is also possible that CCP forms some type of crystalline structure (Horne *et al.*, 2007). Various researchers have included casein (serine) phosphate groups in the calculation of the ratio of Ca/P in CCP and have obtained a value <1.3. It is now realized that phosphoserine groups are not part of CCP but a small number of these peptide residues (around four) can terminate the growth of CCP crystals (Horne *et al.*, 2007).

The pK_a values for phosphate reported in chemistry textbooks are 2.1, 6.9 and 12.0, and Walstra and Jenness (1984) suggested that comparing the pH of milk (~6.7) with the pK_{a2} of phosphoric acid, one would expect $CaHPO_4$ to be the form of CCP. However, in the presence of Ca, all phosphates (pK_{a2} and pK_{a3}) are titrated around pH 6–7 due to the precipitation of Ca phosphate (Figure 9.3). That observation and the strong buffering at pH ~5.1 during acid titration of milk (Figure 9.2a) caused by the protonation of the released phosphate ions (from CCP) suggest that the form of CCP in milk is less likely to be an acidic form (like $CaHPO.2H_2O$) and may be a more basic form (e.g. tricalcium phosphate). Other titration studies, including those with oxalate (Pyne and Ryan, 1950; Jenness, 1973) have indicated that most P_i in CCP is in the form of PO_4^{3-} (i.e. tricalcium phosphate). Holt (1985) suggested that there may be a difficulty in titration studies if it is assumed that the exposed phosphoserine groups, after the dissolution of CCP, do not contribute to the titration. This suggestion by Holt (1985) does not explain the acid–base buffering behaviour shown in Figure 9.2a as the back-titration with base indicated that CCP does indeed contribute to the buffering at pH ~5. Removal of CCP resulted in the elimination of the buffering peak at pH ~5 (Figure 9.2b). Any calculation of milk salts equilibria needs to take into account the unexpected pK_{a2} and pK_{a3} values of phosphate in milk/serum although this does not appear to have always been the case. In summary, evidence from the various types of titration, the real pK_a values for phosphate in milk and the distinctive acid–base buffering properties of milk all suggest that the form of CCP is a basic form (e.g. tricalcium phosphate).

9.7. Functional Properties of Milk Products

Milk salts greatly influence the functional properties of milk and various dairy products primarily by influencing the structural integrity of the casein micelles or the sensitivity to aggregation of caseins. There have been a number of reviews on the effects of salts on the functionality of milk products (e.g. Augustin, 2000).

9.7.1. Rennet-Induced Gels

Generally, it is thought that Ca does not directly affect the enzymatic phase, although addition of $CaCl_2$ does reduce milk pH, which accelerates the hydrolysis reaction (Lucey and Fox, 1993). Rennet-altered micelles will aggregate only in the presence of Ca^{2+}, and gelation occurs only if there is sufficient CCP present (i.e. needs some type of casein micellar structure, as sodium caseinate does not form a rennet-induced gel, even though there is release of the macropeptide). Addition of ($<$50 mM) Ca reduces the rennet coagulation time, even at a constant milk pH, and flocculation occurs at a lower degree of κ-casein hydrolysis. Addition of Ca increases the rate of firming of renneted milk gels, mainly by neutralization of the negatively charged groups on the micelle surface and possibly by the formation of Ca bridges. Addition of high concentrations of Ca (e.g. $>$0.1 M) reduces the rate of gel firming, probably by increasing the effective (positive) surface charge on the micelles. Addition of up to 10 mM Ca increases the strength of rennet-induced gels (Lucey and Fox, 1993). Low levels ($\leq$0.02%) of $CaCl_2$ are often added by cheesemakers to help standardize the coagulation process (e.g. cutting time).

Reduction of the CCP content of casein micelles by $\sim$30% prevents coagulation unless [Ca^{2+}] is increased (Shalabi and Fox, 1982). Udabage *et al.* (2001) investigated the effects of mineral salts and Ca sequestrants or chelating agents on the gelation of renneted skim milk. They found that depending on the level of chelating agent, addition of citrate or ethylenediaminetetraacetic acid (EDTA) reduced the storage modulus (G′) of rennet-induced gels and above a certain concentration rennet gelation was inhibited completely (10 mmol/kg milk).

Choi *et al.* (2007) demonstrated that the concentration of insoluble Ca phosphate (CCP) associated with the casein micelles had an important influence on the properties of rennet-induced gels. Removal of some CCP from milk prior to gelation using a Ca-chelator lowered the storage modulus of rennet-induced gels due to the reduction in the amount of CCP crosslinking in casein micelles. Reduction in the CCP content prior to rennet-induced gelation resulted in gels with higher loss tangent values, indicating greater bond mobility.

The swelling, hydration and solubility of casein micelles in renneted milk are greatly increased in the presence of NaCl but markedly reduced if the brine solution contains Ca (Lucey and Fox, 1993). The addition of high concentrations of NaCl causes a reduction in rennet coagulation time. In some cheese varieties, salt is added to the cheesemilk (e.g. Domiati) resulting in a slower set and weaker curd (Fahmi and Shahara, 1950). These changes are largely reversible on removal of the excess NaCl by exhaustive dialysis against bulk milk (Huppertz, 2007).

9.7.2. Acid-Induced Milk Gels

Acid-induced casein gels can be made from sodium caseinate, indicating that the presence of CCP is not a requirement for the formation of acid milk gels (Lucey *et al.*, 1997). Since CCP is completely soluble at pH $\leq$ 5, CCP crosslinks do not contribute to the (final, or at least at pH values <5) stiffness of acid milk gels. The rate and extent of CCP solubilization during the gelation process is an important variable influencing acid milk gel properties.

The addition of Ca-chelating agents to milk has been reported to increase the firmness of acid milk gels made with glucono-δ-lactone (GDL) (Johnston and Murphy, 1992). Addition of EDTA also caused an increase in the loss tangent (tan δ) value in acid-heat-induced skim milk gels (Goddard and Augustin, 1995). Recently, Ozcan-Yilsay *et al.* (2007) studied the effect of trisodium citrate (TSC) on the rheological and physical properties and microstructure of yogurt. The storage modulus of gels increased significantly on addition of low levels of TSC and highest values were observed in samples with 10–20 mM TSC; higher (>20 mM) concentrations of TSC resulted in a large decrease in stiffness. No maximum in tan δ was observed in yogurts made with $\geq$25 mM of TSC as CCP was dissolved completely prior to gelation. Partial removal of CCP resulted in an increase in the tan δ at pH 5.1. Ozcan-Yilsay *et al.* (2007) suggested that at low TSC levels, the removal of CCP crosslinks may have facilitated greater rearrangement and molecular mobility of the micelle structure, which may have helped to increase the storage modulus and tan δ of gels by increasing the formation of crosslinks between strands. Ozcan-Yilsay *et al.* (2007) also concluded that the tan δ maximum observed in yogurts made from heated milk was due to the presence of CCP, as modification of the CCP content altered this peak and the removal of CCP eliminated this feature in the tan δ profiles.

Roefs and van Vliet (1990) reported that increasing the concentration of NaCl added to cold-acidified skim milk samples resulted in a decrease in the dynamic moduli of the gels formed when these samples were warmed. This indicated that electrostatic interactions are important for particle interactions. At high ionic strength, charged groups on casein particles would be screened, thereby weakening interactions between particles, which would result in a slower rate of increase of the storage moduli. In preparing Na caseinate gels by cold acidification, the addition of at least 0.1 M NaCl (to the acidified sample) was necessary to prevent precipitation during the warming up procedure (Roefs and van Vliet, 1990). Possibly, the primary effect of NaCl was to reduce rearrangement during the aggregation stage of gel formation. The addition of a high concentration of NaCl (>0.24 mol L^{-1}) to cold-acidified milk prevented gel formation when it was subsequently heated to a higher temperature for gelation (Roefs and van Vliet, 1990). Lucey *et al.*

(1997) studied the impact of NaCl on the properties of acid casein gels. They found that the pH at gelation was lower, $\leq$5.0, in gels made with added NaCl than in gels made without added NaCl, pH $\sim$ 5.1.

Low-methoxyl pectin is often used as a stabilizer in acid milk gel systems. Harte *et al.* (2007) proposed that during the acidification of milk, the release of Ca^{2+} arising from the solubilization of CCP induces the formation of pectin–pectin complexes, and at lower pH values these complexes interact with the casein particles. For acid casein gels made in the absence of Ca ions, a substantial reduction in the storage modulus was detected at pectin concentrations as low as 0.01–0.02% (w/v) and a significant increase in gelation time at pectin concentrations $\geq$0.05% (w/v) (Matia-Merino *et al.*, 2004). Complete inhibition of acid-induced gelation of casein was noted at $\geq$0.8% (w/v) pectin. Addition of Ca at low pectin contents (<0.2%) reduced the modulus of acid milk gels but there was a large increase in the storage modulus at higher levels of pectin ($\geq$0.2%, w/v).

9.7.3. Heat-Induced Whey Protein Gels

Salts have a major effect on the type, as well as the mechanical/sensory properties, of whey protein gels formed as a result of heat treatment. It is generally recognized that the addition of $CaCl_2$ to dialysed samples of whey protein concentrate (WPC) or whey protein isolate (WPI) results in an increase in gel strength. Above a level of 10–20 mM $CaCl_2$ gel firmness starts to decrease (Schmidt *et al.*, 1979; Kuhn and Foegeding, 1991). It has been speculated that excessive Ca causes rapid protein aggregation (due to decreased protein stability), which limits protein unfolding and network formation (Mangino, 1992). Caussin *et al.* (2003) reported that the addition of Ca to whey proteins resulted in the formation of very large protein aggregates during heating. Most commercially available WPC products probably have a Ca content that is greater than that required for optimal gel strength (Mangino, 1992). There is considerable variability in the thermal aggregation behaviour of commercial whey products and some of these differences could be removed by dialysis of these samples to a common ionic strength (McPhail and Holt, 1999). The concentrations of divalent cations are higher in WPC made from cheese whey than in WPC made from acid whey and these cations are not easily removed by dialysis, suggesting some binding by the whey proteins (Havea *et al.,* 2001). Presumably, membrane filtration of acid whey WPC at low pH values resulted in its greater demineralization. WPC made from acid whey has superior heat gelling properties than WPC made from rennet-coagulated cheese whey (Veith and Reynolds, 2004). These differences could be due to absence of GMP and the low Ca concentration in acid whey WPC.

9.7.4. Cold-Set Whey Protein Gels

Whey protein gels can also be produced using a two-step process that involves heat treatment at low ionic strength and/or far from the isoelectric point, followed by an increase in ionic strength and/or adjustment of pH (Barbut and Foegeding, 1993; Britten and Giroux, 2001). These gels are called cold-set gels, as the initial heat treatment produces a polymerized solution and gelation can occur at low temperatures ($\leq$ambient) if the repulsive forces are screened by the addition of mono- or polyvalent cations (e.g. Ca^{2+}) or a decrease in pH (e.g. through the addition of GDL or by bacterial fermentation). To obtain gels via the cold-set gelation method, it is necessary to first prepare a solution of heat-denatured proteins, with a protein concentration below the critical gelation concentration. Heating (e.g. 80°C for 30 min) results in the formation of soluble, denatured whey protein aggregates. Whey protein fibril-type gels are formed at very low pH values (e.g. 2) and cold-set fibril gels can also be made by the addition of Ca^{2+} (Bolder *et al.*, 2006).

9.7.5. Emulsions

Caseins, especially caseinates, are widely used as emulsifiers (Dickinson, 1997). The aggregation state of casein greatly influences surface activity with sodium caseinate (non-micellar), having greater surface activity than micellar or Ca caseinate (Mulvihill and Murphy, 1991). Dalgleish (1987) reported that emulsions prepared with α_s- or β-casein were sensitive to precipitation by Ca but emulsions prepared with κ-casein did not aggregate on Ca addition. The phosphoserine residues in β-casein helped that molecule maintain a thick steric stabilizing monolayer on emulsion interfaces (Dickinson, 1997). Increasing ionic strength by the addition of electrolytes screens out the double-layer repulsion and therefore reduces the electrostatic stabilization of proteins. Therefore, emulsions prepared with commercial milk protein ingredients of high salt content may be more flocculated than model systems prepared with pure proteins dissolved in low ionic strength buffer solutions (Dickinson, 1997). Calcium ions influence the stability of sodium caseinate-stabilized emulsions (Ye and Singh, 2001). Addition of $CaCl_2$ before or after homogenization caused a decrease in the creaming stability of emulsions made with 0.5% caseinate. In contrast, addition of $CaCl_2$ up to $\sim$10 mM increased the creaming stability of emulsions made with 3% caseinate, although the stability decreased again $>$20 mM $CaCl_2$. There was an increase in the surface protein concentration with an increase in the level of $CaCl_2$, which was due to enhanced adsorption of the α_s-caseins (Ye and Singh, 2001).

9.7.6. Foaming and Rehydration Properties After Spray Drying

Milk exhibits improved foam expansion when treated with EDTA (Ward *et al.*, 1997), probably due to disruption of the micellar structure following the chelation of CCP. The Ca concentration influences the interactions of β-casein at the air–water interface; in the absence of Ca, a weak interfacial gel forms whereas with Ca addition, a strong interfacial gel forms quickly (Vessely *et al.*, 2005). The foamability of reconstituted skim milk powder increased as the NaCl concentration was increased from 0 to 0.8 M due to the gradually increasing dissociation of casein micelles (Zhang *et al.*, 2004). The foamability of whey protein isolate increased when NaCl concentration was increased from 0 to 0.1 M, but decreased at higher NaCl concentrations (Zhang *et al.*, 2004).

The addition of citrate or phosphate solutions to micellar casein suspensions before drying considerably increased rehydration rates and this was related to the destruction of the micelle structure (Schuck *et al.*, 2002); this also alters the calcium equilibrium as extensive washing removes most soluble calcium. Water uptake in casein suspensions was improved by adding NaCl during rehydration. The addition of $CaCl_2$ considerably affected micelle organization and led to the formation of insoluble structures during spray drying.

9.7.7. Stability of Caseins

9.7.7.1. Ethanol

The stability of milk to various concentrations of added ethanol has been used as a milk quality index and is important in the production of drinks, such as cream liqueurs. Figure 9.5 is an attempt to illustrate the impact of pH on the ethanol and heat stability of caseins. Low pH values reduce stability and stability increases sigmoidally with pH. The inflection point (pK) depends on the properties of individual milks. For a more complete description of this profile/behaviour see Horne (2003). Horne and Parker (1981) found that the addition of Ca or Mg to milk samples caused a shift of the ethanol stability (ES)/pH profile to more alkaline pH. The addition of phosphate or citrate had little or no effect on the ES/pH profile, although addition of the stronger sequestrant, EDTA, caused a shift in the profile to more acidic pH. The studies of Horne and Parker on ethanol stability, reviewed by Horne (2003), emphasized the role of the inorganic components of the milk serum, reinforcing the conclusions of Sommer and Binney (1923) that salt balance, the excess of Ca and Mg over citrate and phosphate in milk serum, was critical in alcohol-induced coagulation. Decreasing the salt balance ratio thus caused a shift in the ES/pH profile to acidic pH, whereas increasing the salt balance ratio shifted the profile to more alkaline values. The mechanism, proposed by Horne (1987) to explain these observations, suggests that ethanol has two

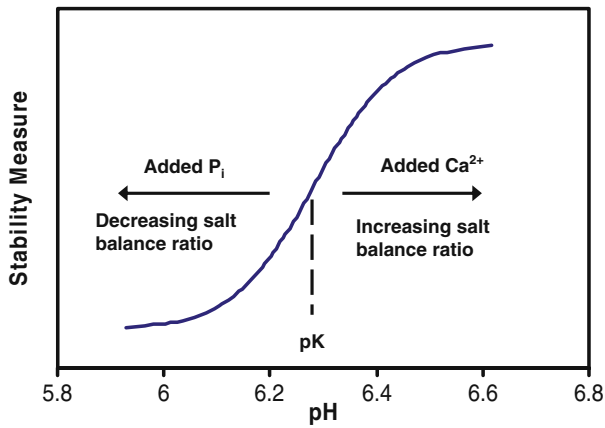

Figure 9.5. Stability phase diagram of milk as a function of pH or other treatments; schematic, meant to indicate trends for both ethanol and heat stability behaviour of milk (adapted from Horne, 2003).

competing effects on the micellar system, destabilization through loss of the hairy layer and shifts in the Ca phosphate equilibria, first noted by Pierre (1985). If the ethanol promotes the precipitation of Ca phosphate external to the micelle, it would first reduce the concentration of free Ca, reduce the level of caseinate-bound Ca and disrupt the binding through Ca phosphate nanoclusters. Moderate losses would increase the negative charge on the caseins and increase the thickness of the steric stabilizing layer. The higher the alcohol concentration, the faster and more extensive would be the precipitation of Ca phosphate. The ensuing adjustment in protein charge and conformation, although relatively rapid, still requires a finite response time. Countering these changes are the effects of ethanol as a non-solvent for the proteins, promoting crosslinking and collapse of the hairy layer. When the coagulation reaction occurs faster than the adjustment of charge and conformation resulting from shifts in Ca phosphate equilibria or the extent of the latter is limited by insufficient ethanol, the aggregation reaction dominates and precipitation of micelles follows.

The origin of the sigmoidal ethanol stability/pH profile (Figure 9.5) can also be explained through the effect of pH on Ca phosphate precipitation. Increasing pH brings about increased Ca phosphate precipitation, possibly further enhanced by the ethanol, which means that more ethanol is required to precipitate the protein, i.e. to overcome the increased energy barrier being erected following the transfer of Ca phosphate from the nanoclusters. Conversely, decreasing pH acts to diminish the influence of ethanol-induced

precipitation of Ca phosphate by titrating away negative charge and reducing electrostatic repulsion between protein species. Other effects of milk serum composition, of forewarming the milk and of modifying milk concentration and ionic strength, can all be explained in a similar fashion (Horne, 2003).

Tsioulpas *et al.* (2007) reported that there is an inverse non-linear relationship between free Ca ion concentration and ethanol stability ($r = 0.84$), confirming the earlier observations of Davies and White (1958). Citrate in natural samples acts as a stabilizing factor, as it slightly improved milk stability (Tsioulpas *et al.*, 2007). Ethanol stability values for milks during lactation were reported to have a mean of 83.2 ± 12.6% (range 62–100%) (Tsioulpas *et al.*, 2007). Chavez *et al.* (2004) found a (positive) correlation between the concentrations of chloride, potassium, ionic Ca and somatic cell count and ethanol stability. Horne and Parker (1983) reported that the addition of NaCl reduced the ethanol stability of unconcentrated milk, primarily at pH > 6.5, which they suggested is a result of increased ionic strength. O'Kennedy *et al.* (2001) demonstrated that α_{s1}- and β-caseins were only minor components of the ethanol-induced precipitate whereas α_{s2}- and κ-casein were the main proteins susceptible to aggregation.

9.7.7.2. Heat

Heat stability of milk has been reviewed (Fox and Morrissey, 1977; Singh and Creamer, 1992; O'Connell and Fox, 2003; Singh, 2004). Older literature was reviewed by Pyne (1962). The effects of raising temperature on the status of the various Ca phosphate species have been discussed earlier (Section 9.4.1). Heat stability is the ability of milk or concentrates to resist severe heat treatments without thickening, gelation or coagulation (Augustin, 2000).

Discussions on heat stability are complicated by the knowledge that heat-induced coagulation as a function of pH can follow two distinct profiles. In the Type A heat coagulation time (HCT) versus pH profile, the time to induce coagulation at a fixed temperature first increases with pH, then enters a minimum before stability increases again at more alkaline pH values. In the Type B profile, heat coagulation time increases progressively with pH. Individual milks which follow Type A behaviour predominate in most countries, while all bulk milks show Type A behaviour (O'Connell and Fox, 2003). When milk is heated, several competitive and often interdependent reactions occur, not all of them directly involving the milk salts. Fox (1981) listed a selection of these but it is now generally agreed that the presence of the minimum in a Type A profile is associated with the heat-induced formation of a complex between β-lactoglobulin and κ-casein. Such chemical reactions

are outside the scope of this chapter and are covered in the reviews of Fox and Morrissey (1977), Singh and Creamer (1992), O'Connell and Fox (2003) and Singh (2004). However, milks showing Type A characteristics can be converted into Type B profiles and vice versa. For a list of methods and a discussion of these observations, see Horne and Muir (1990). Interestingly, several of these methods involve manipulating the levels of milk salts, particularly Ca and phosphate. For many years, it was considered that differences in the heat stability of milk were due to variations in the composition of milk salts and this led Sommer and Hart (1919) to propose the salt balance theory referred to above in our discussion of ethanol stability. O'Connell and Fox (2003) have suggested that subsequent attempts to correlate heat stability with natural variations in the composition of milk salts failed because the original studies were based on deliberate additions of salts to milk at levels outside natural variability. This overlooks the fact that the experiments of Sommer and co-workers employed a different protocol for the heat stability assay, namely a measurement of the heat coagulation temperature, the temperature at which milk instantaneously coagulates (i.e. effectively coagulates within a short time, <2 min). Because it is a measure of instantaneous coagulation, it is unaffected by changes that occur on prolonged heating. Instead, the response to changing pH, as observed by Miller and Sommer (1940), is remarkably similar to the sigmoidal ethanol stability/pH profile. Moreover, the addition of Ca shifts this profile to more alkaline values while the addition of phosphate has the opposite effect of producing an acidic shift, just like the response of ethanol stability profiles. Horne and Muir (1990) suggested that such behaviour indicated that heat-induced coagulation as measured by this assay might follow a similar, if not identical, pathway to alcohol-induced coagulation as described above, involving the precipitation of Ca phosphate and a decrease in Ca activity with increasing pH. Such a scenario also ties in with the observation that the amount of free Ca^{2+} has been associated by various authors with the heat stability of milk, powdered milk and recombined milk (Augustin and Clarke, 1990; Singh and Creamer, 1992; Williams *et al.*, 2005). Addition of Ca to milk results in a decrease in heat stability due to the increase in free [Ca^{2+}] (Philippe *et al.*, 2004). Seasonal changes in milk salts (soluble Ca) have been correlated with the heat stability of milk (Kelly *et al.*, 1982). Salts, such as orthophosphates, are often added to milk concentrates (or ultra-high-temperature sterilized milks) during processing to improve heat stability. Orthophosphates reduce the Ca^{2+} activity, which is mainly responsible for the improved heat stability (Augustin and Clarke, 1990). O'Connell and Fox (2001) suggested that heat-induced precipitation of CCP is involved in the thermal coagulation of milk and that the specific effect of β-lactoglobulin at the pH of maximum stability may be related to its ability to chelate Ca.

9.7.8. Cheese Texture and Functionality

The importance of calcium and phosphate interactions for cheese manufacturing properties, as well as the textural properties, has been reviewed (Lucey and Fox, 1993; McMahon and Oberg, 1998; Lucey *et al*., 2003; Johnson and Lucey, 2006). The process cheese industry is based on the use of citrate or phosphate salts to sequester some of the Ca from the residual CCP, which solubilizes caseins that can then emulsify fat globules. The acidity of whey at drainage and rate of acid development are recognized as important parameters that determine the mineral content, acidity and quality of cheese. Schulz (1952) developed a classification of cheese varieties based on their Ca contents. Monib (1962) was one of the first investigators to study the Ca phosphate–casein complex in cheese and he concluded that very dilute cheese extracts did not represent cheese-like conditions and their use would lead to incorrect conclusions about serum Ca concentrations (i.e. excessive dilution resulted in the dissolution of more insoluble Ca). By the 1980s, it was recognized that acid development during manufacture determines the loss of Ca, which determines the basic structure of cheese (e.g. Lawrence *et al*., 1983). By the early 1990s, there was the realization that much of the residual Ca in cheese is associated with casein and that much of the CCP was not dissolved during cheesemaking (Lucey and Fox, 1993). It was also recognized that the residual insoluble Ca component is an important structural unit influencing cheese texture (Lucey and Fox, 1993). Many studies have demonstrated the importance of pH and Ca content on the functional properties of cheese (e.g. Yun *et al*., 1993; McMahon and Oberg, 1998; Guinee *et al*., 2002; Joshi *et al*., 2002). It is now accepted that during ripening there are important changes in the amount of insoluble Ca (e.g. Guo and Kindstedt, 1995; Hassan *et al*., 2004) and that these shifts in the Ca equilibrium contribute to textural changes during ripening (Lucey *et al*., 2005; O'Mahony *et al*., 2005). The proportion of insoluble Ca in cheese has been estimated by the expression of some of the aqueous phase ("juice") under high hydraulic pressure (Morris *et al*., 1988; Lucey and Fox, 1993), centrifugation to extract some expressible serum in young, high-moisture cheeses (Guo and Kindstedt, 1995), acid–base buffering (Lucey and Fox, 1993; Hassan *et al*., 2004) and water extraction methods (Metzger *et al*., 2001).

9.8. Other Uses/Applications of Milk Salts

Milk minerals (typical composition: <5% protein, <9% lactose, >70% ash, 25% Ca, 14% phosphorus) are produced by concentrating and drying deproteinized delactosed whey. This ingredient is often used for mineral

fortification purposes in a range of food products. A number of biologically active peptides are released during digestive breakdown of caseins and they play a physiological role in newborn mammals (Kitts, 2006). Casein phosphopeptides (CPP) are resistant to further hydrolysis by mammalian digestive enzymes and accumulate in the small intestine. CPP renders Ca^{2+} in a relatively soluble form for a potential enhanced bioavailability by paracellular (passive) mechanisms. CPPs are produced commercially by a number of dairy companies and used as a nutritional ingredient to enhance mineral absorption as well as provide anticariogenic benefits (Reynolds, 1999; Tsuchita *et al.*, 2001).

9.9. Concluding Remarks

Milk salts play a critical role in the formation and stability of casein micelles. Milk salts influence many of the important functional properties of milk products including gelation, protein stability, emulsification, foaming and cheese texture. The concentration of milk salts can be varied by processing conditions including acidification or the addition of metal chelators/sequestrants. The nature and structure of CCP is still under debate. The manipulation of the amount of insoluble Ca in cheese is the major focus of ongoing studies related to controlling cheese performance. There is growing awareness of the nutritional benefits of Ca and P, which has resulted in the fortification of many dairy products like cheese with Ca.

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