5 Salts of milk

5.1 Introduction

The salts of milk are mainly the phosphates, citrates, chlorides, sulphates, carbonates and bicarbonates of sodium, potassium, calcium and magnesium. Approximately 20 other elements are found in milk in trace amounts, including copper, iron, silicon, zinc and iodine. Strictly speaking, the proteins of milk should be included as part of the salt system since they carry positively and negatively charged groups and can form salts with counter-ions; however, they are not normally treated as such. There is no lactate in freshly drawn milk but it may be present in stored milk and in milk products. The major elements are of importance in nutrition, in the preparation, processing and storage of milk products due to their marked influence on the conformation and stability of milk proteins, especially caseins, and to a lesser extent the stability of lipids and the activity of some indigenous enzymes.

5.2 Method of analysis

The mineral content of foods is usually determined from the ash prepared by heating a sample at 500–600°C in a muffle furnace for about 4 h to oxidize organic matter. The ash does not represent the salts as present in the food because:

1. the ash is a mixture, not of the original salts, but of the carbonates and oxides of the elements present in the food;
2. phosphorus and sulphur from proteins and lipids are present in the ash, while organic ions, such as citrate, are lost during incineration; and
3. the temperature usually employed in ashing may vaporize certain volatile elements, e.g. sodium and potassium.

Therefore, it is difficult or impossible to relate the ash obtained from a food with its salts system, and low values are obtained for certain mineral elements by analysis of the ash compared to direct analysis of the intact food. Titrimetric, colorimetric, polarographic, flame photometric and atomic absorption spectrophotometric techniques are frequently used to analyse for the various mineral constituents; however, the quantitative estimation of

each ion in a mixture is frequently complicated by interfering ions. The major elements/ions in foods, including milk, may be determined by the following specific methods:

- Inorganic phosphate reacts with molybdate to form phosphomolybdate which may be reduced to a blue compound that can be quantified spectrophotometrically at 640 nm.
- Calcium and magnesium may be determined by titration with EDTA or by atomic absorption spectroscopy on TCA filtrates or on wet- or dry-ashed samples.
- Citrate forms a yellow complex with pyridine (which is carcinogenic) in the presence of acetic anhydride; the complex may be quantified spectrophotometrically. Alternatively, citrate can be determined by an enzymatic assay.
- Ionized calcium may be determined spectrophotometrically after reaction with murexide or using a Ca^{2+}-specific electrode.
- Sodium and potassium may be quantified by flame photometry, atomic absorption spectroscopy or ion specific electrodes.
- Chloride can be titrated with $AgNO_3$ using potentiometric or indicator end-point detection.
- Sulphate is precipitated by $BaCl_2$ and quantified gravimetrically.
- Lactate may be quantified spectrophotometrically after reaction with $FeCl_2$, or by an enzymatic assay (using lactate dehydrogenase which can quantify both D- and L-isomers) or by HPLC.

References to these and other methods can be found in Jenness (1988). Detailed analytical procedures are published in the *Official Methods of Analysis of the Association of Official Analytical Chemists* (Arlington, VA, USA) or in *Standard Methods of the International Dairy Federation* (Brussels, Belgium).

5.3 Composition of milk salts

The ash content of milk remains relatively constant at 0.7–0.8%, but the relative concentrations of the various ions can vary considerably. Table 5.1 shows the average concentration of the principal ions in milk, the usual range and the extreme values encountered. The latter undoubtedly include abnormal milks, e.g. colostrum, very late lactation milk or milk from cows with mastitic infection.

The ash content of human milk is only about 0.2%; the concentration of all principal and several minor ions is higher in bovine than in human milk (Table 5.2). Consumption of unmodified bovine milk by human babies causes increased renal load and hence demineralized bovine milk or whey should be used for infant formulae.

Table 5.1 Concentration of milk salt constituents (mg litre^{-1} milk (from various sources)

Constituent	Average content	Usual range	Extremes reported
Sodium	500	350–600	110–1150
Potassium	1450	1350–1550	1150–2000
Calcium	1200	1000–1400	650–2650
Magnesium	130	100–150	20–230
Phosphorus (total)[a]	950	750–1100	470–1440
Phosphorus (inorganic)[b]	750		
Chloride	1000	800–1400	540–2420
Sulphate	100		
Carbonate (as CO_2)	200		
Citrate (as citric acid)	1750		

[a]Total phosphorus includes colloidal inorganic phosphate, casein (organic) phosphate, soluble inorganic phosphate, ester phosphate and phospholipids.
[b]Phosphorus (inorganic) includes colloidal inorganic phosphate and soluble inorganic phosphate.

Table 5.2 Mineral composition (mg or $\mu g\,l^{-1}$) of mature human or bovine milks (from Flynn and Power, 1985)

	Mature human milk		Cows' milk	
Constituent	Mean	Range	Mean	Range
Sodium (mg)	150	110–200	500	350–900
Potassium (mg)	600	570–620	1500	1100–1700
Chloride (mg)	430	350–550	950	900–1100
Calcium (mg)	350	320–360	1200	1100–1300
Magnesium (mg)	28	26–30	120	90–140
Phosphorus (mg)	145	140–150	950	900–1000
Iron (μg)	760	620–930	500	300–600
Zinc (μg)	2950	2600–3300	3500	2000–6000
Copper (μg)	390	370–430	200	100–600
Manganese (μg)	12	7–15	30	20–50
Iodine (μg)	70	20–120	260	–
Fluoride (μg)	77	21–155	–	30–220
Selenium (μg)	14	8–19	–	5–67
Cobalt (μg)	12	1–27	1	0.5–1.3
Chromium (μg)	40	6–100	10	8–13
Molybdenum (μg)	8	4–16	73	18–120
Nickel (μg)	25	8–85	25	0–50
Silicon (μg)	700	150–1200	2600	750–7000
Vanadium (μg)	7	Tr–15	–	Tr–310
Tin (μg)	–	–	170	40–500
Arsenic (μg)	50	–	45	20–60

Tr, Trace.

5.4 Secretion of milk salts

The secretion of milk salts, which is not well understood, has been reviewed and summarized by Holt (1985). Despite the importance of milk salts in determining the processing characteristics of milk, relatively little interest has been shown in the nutritional manipulation of milk salts composition.

Three factors must be considered when discussing the milk salts system:

1. the need to maintain electrical neutrality;
2. the need to maintain milk isotonic with blood; as a result of this, a set of correlations exist between the concentrations of lactose, Na^+, K^+ and Cl^-;
3. the need to form casein micelles which puts constraints on the pH and $[Ca^{2+}]$ and requires the complexation of calcium phosphate with casein.

Skim milk can be considered as a two-phase system consisting of casein–colloidal calcium phosphate micelles in quasi-equilibrium with an aqueous solution of salts and proteins; the phase boundary is ill-defined because of the intimate association between the calcium phosphate and the caseins (phosphoproteins).

A fat-free primary secretion is formed within vesicles formed by blebbing-off of the Golgi dicytosomes; the vesicles pass through the cytoplasm to the apical membrane where exocytosis occurs. The vesicles contain casein (synthesized in the rough endoplasmic reticulum toward the base of the mammocyte); fully-formed casein micelles have been demonstrated within the Golgi vesicles. The vesicles also contain lactose synthetase (UDP:galactosyl transferase and α-lactalbumin) and there is good evidence showing that lactose synthesis occurs within the vesicles from glucose and UDP-galactose transported from the cytosol.

The intracellular concentrations of sodium and potassium are established by a Na^+/K^+-activated ATPase and Na^+ and K^+ can permeate across the vesicle membranes. Calcium is probably necessary to activate the UDP:galactosyl transferase and is transported by a Ca^{2+}/Mg^{2+}-ATPase which concentrates Ca^{2+} against an electrical potential gradient from μM concentrations in the cytosol to mM concentrations in the vesicles. Inorganic P (P_i) can be formed intravesicularly from UDP formed during the synthesis of lactose from UDP-galactose and glucose. UDP, which cannot cross the membrane, is hydrolysed to UMP and P_i, both of which can re-enter the cytosol (to avoid product inhibition); however, some of the P_i is complexed by Ca^{2+}. Ca^{2+} are also chelated by citrate to form largely soluble, undissociated complexes and by casein to form large colloidal casein micelles.

Water movement across the vesicle membranes is controlled by osmotic pressure considerations. Since lactose is a major contributor to the osmotic pressure of milk, the concentrations of both soluble and colloidal salts in

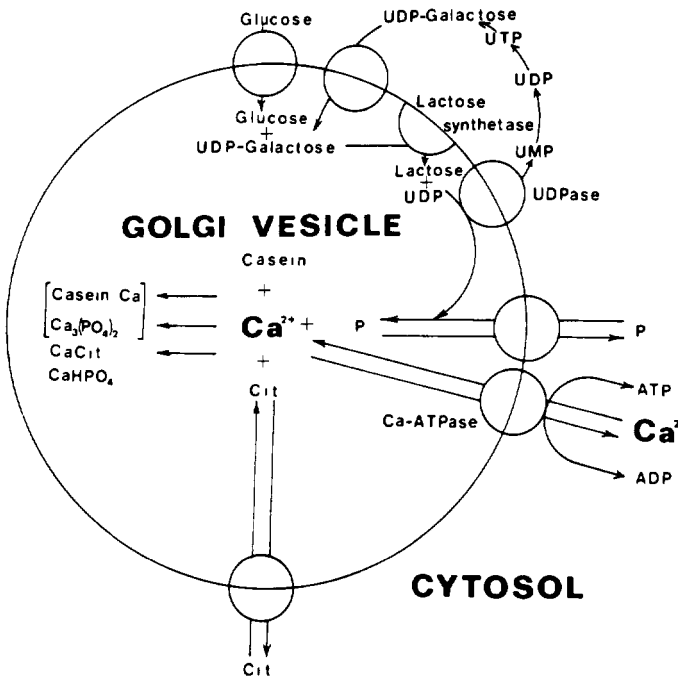


Figure 5.1 Summary of some transport mechanisms for calcium, phosphate and citrate from the cytosol of the secretory cell to the inside of Golgi vesicles (from Holt, 1981).

milk are strongly influenced by lactose concentration and the mechanism by which it is synthesized.

Inter-relationships in the biosynthesis of the principal milk salts are summarized in Figure 5.1. Transport of several ionic species via the junctions between cells (paracellular) occurs during early and late lactation and during mastitic infection when the junctions between cells are more open.

5.5 Factors influencing variation in salt composition

The composition of milk salts is influenced by a number of factors, including breed, individuality of the cow, stage of lactation, feed, mastitic infection and season of the year. The more important factors are discussed below.

5.5.1 Breed of cow

Milk from Jersey cows usually contains more calcium and phosphorus than milk from other breeds, including Holstein, but the concentrations of sodium and chloride are usually lower.

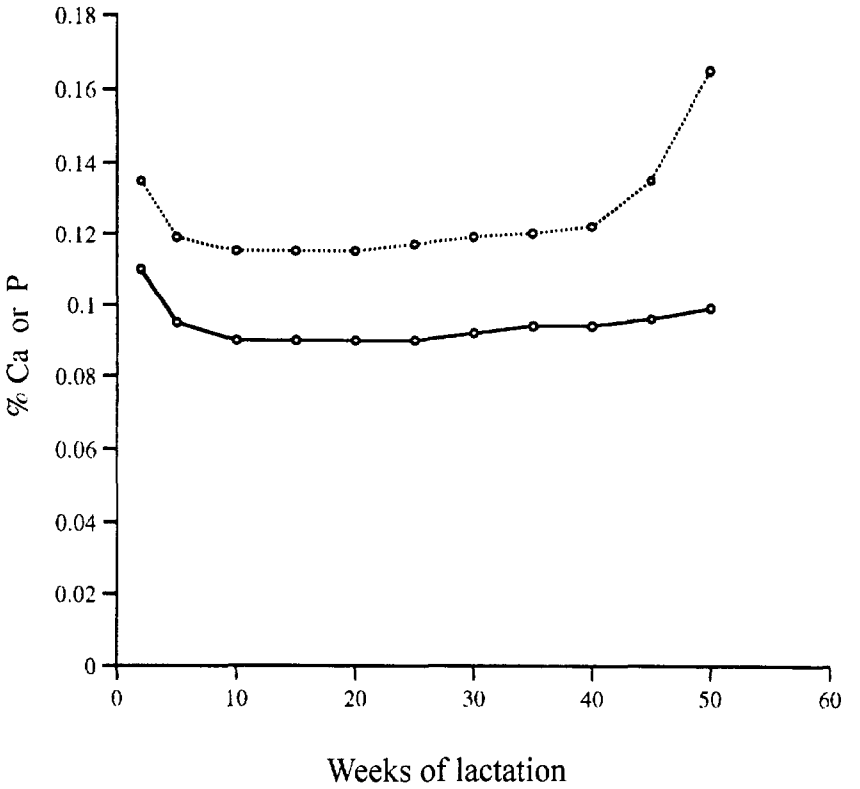


Figure 5.2 Changes in the concentrations of calcium (----) and phosphorus (—) in bovine milk during lactation.

5.5.2 Stage of lactation

The concentration of total calcium is generally high both in early and late lactation but in the intervening period no relation with stage of lactation is evident (Figure 5.2). Phosphorus shows a general tendency to increase as lactation advances (Figure 5.2). The concentrations of colloidal calcium and inorganic phosphorus are at a minimum in early and at a maximum in late lactation milk. The concentrations of sodium and chloride (Figure 5.3) are high at the beginning of lactation, followed by a rapid decrease, then increase gradually until near the end of lactation when rapid increases occur. The concentration of potassium decreases gradually throughout lactation. The concentration of citrate, which has a marked influence on the distribution of calcium, shows a strong seasonal variation (Figure 5.4), influenced more by feed than the stage of lactation. The pH of milk shows a strong

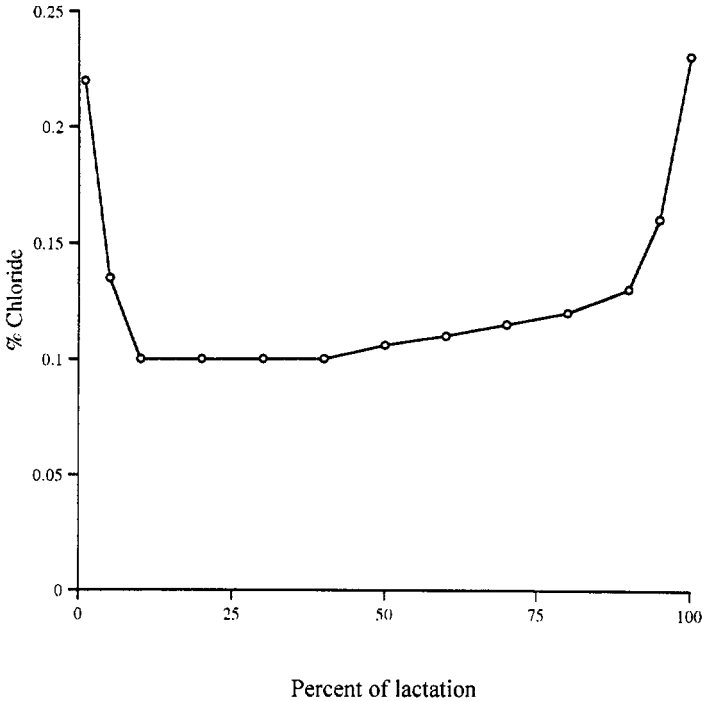


Figure 5.3 Changes in the concentration of chloride in bovine milk during lactation.

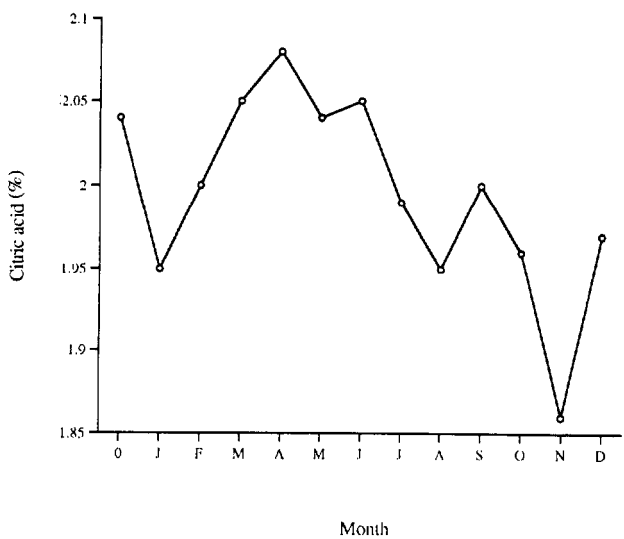


Figure 5.4 Seasonality of the concentration of citric acid in bovine milk.

seasonal trend; the pH of colostrum is about 6 but increases to the normal value of about 6.6–6.7 shortly after parturition and changes little until late lactation, when the pH raises to as high as 7.2, i.e. approaches that of blood (pH 7.4) due to degeneration of the mammary cell membrane. The pH of milk also increases during mastitic infection (e.g. 6.8–6.9), due to the influx of constituents from blood.

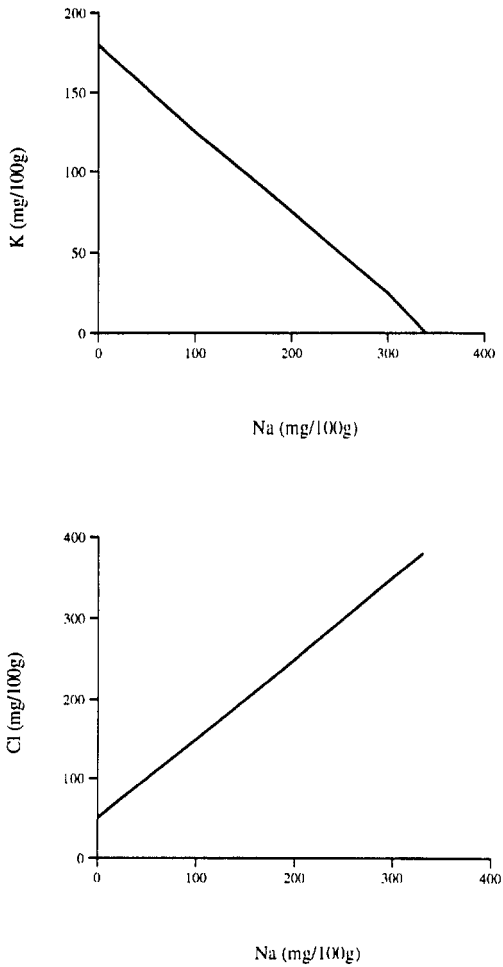


Figure 5.5 Correlations between the concentration of sodium and potassium (a) and sodium and chloride (b) in bovine milk.

5.5.3 Infection of the udder

Milk from cows with mastitic infections contains a low level of total solids, especially lactose, and high levels of sodium and chloride, the concentration of which are directly related (Figure 5.5). The sodium and chloride ions come from the blood to compensate osmotically for the depressed lactose synthesis or vice versa.

These are related by the Koestler number:

$$\text{Koestler number} = \frac{100 \times \%\,\text{Cl}}{\%\,\text{lactose}}$$

which is normally 1.5–3.0 but increases on mastitic infection and has been used as an index of such (better methods are now available, e.g. somatic cell count, activity of certain enzymes, especially catalase and *N*-acetyl-glucosamidase). The pH of milk increases to approach that of blood during mastitic infection.

5.5.4 Feed

Feed has relatively little effect on the concentration of most elements in milk because the skeleton acts as a reservoir of minerals. The level of citrate in milk decreases on diets very deficient in roughage and results in the 'Utrecht phenomenon', i.e. milk of very low heat stability. Relatively small changes in the concentrations of milk salts, especially of Ca, P_i and citrate, can have very significant effects on the processing characteristics of milk and hence these can be altered by the level and type of feed, but definitive studies on this are lacking.

5.6 Interrelations of milk salt constituents

Various milk salts are interrelated and the interrelationships are affected by pH (Table 5.3). Those constituents, the concentrations of which are related to pH in the same way, are also directly related to each other (e.g. the concentrations of total soluble calcium and ionized calcium), while those related to pH in opposite ways are inversely related (e.g. the concentrations of potassium and sodium).

Relationships between some of the more important ions/molecules are shown in Figure 5.6. Three correlations are noteworthy:

1. The concentration of lactose is inversely related to the concentration of soluble salts expressed as osmolarity. This results from the requirement that milk be isotonic with blood.

Table 5.3 Relationships between the pH of milk and the concentrations of certain milk salt constituents

Inversely related to pH	Directly related to pH
Titratable acidity	Colloidal inorganic calcium
Total soluble calcium	Caseinate calcium
Soluble unionized calcium	Colloidal inorganic phosphorus
Ionized calcium	Colloidal calcium phosphate
Soluble magnesium	Sodium
Soluble citrate	Chloride
Soluble inorganic phosphorus	Total phosphorus
Ester phosphorus	
Potassium	

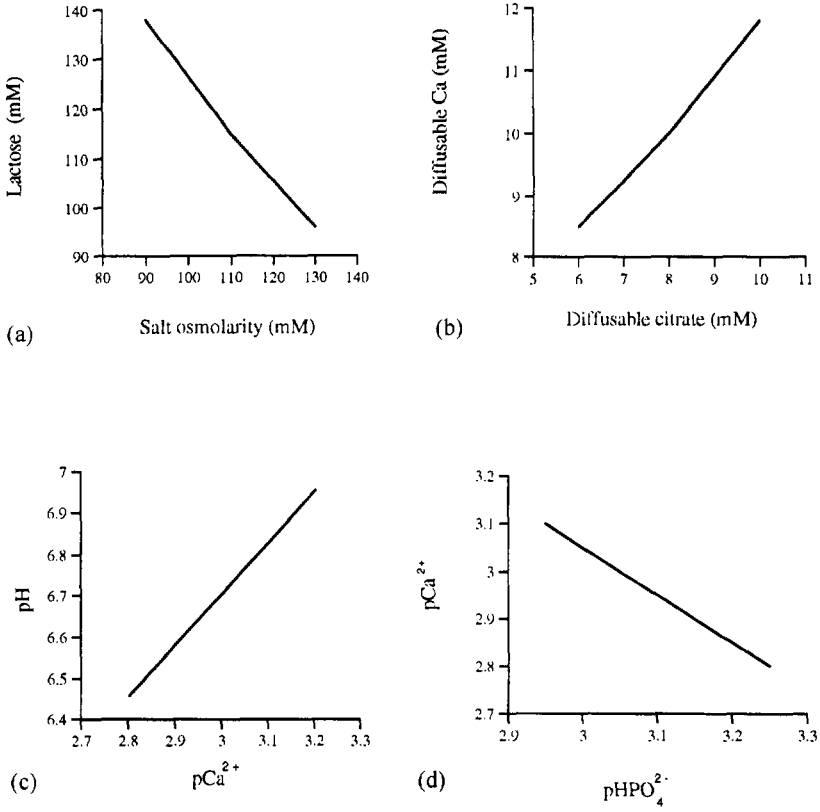


Figure 5.6 Interrelationships between lactose and soluble salts (osmolarity) and between some soluble salts in bovine milk.

2. There is a direct correlation between the concentration of diffusible Ca (and diffusible Mg) and the concentration of diffusible citrate (Figure 5.6b); this correlation, which is very good at constant pH, exists because citrate chelates Ca^{2+} more strongly than phosphate to form soluble unionized salts.
3. The ratio $HPO_4^{2-}/H_2PO_4^-$ is strongly pH dependent, as is the solubility of $Ca_3(PO_4)_2$ (section 5.8.1). As the pH is reduced, colloidal $Ca_3(PO_4)_2$ dissolves but $HPO_4^{2-} \rightarrow H_2PO_4^-$ as the pH is reduced and hence both $[Ca^{2+}]$ and soluble P_i are directly related to pH (Figure 5.6c). The $[HPO_4^{2-}]$ is inversely related to $[Ca^{2+}]$ (Figure 5.6d).

5.7 Partition of milk salts between colloidal and soluble phases

Certain of the milk salts (e.g. chlorides, and the salts of sodium and potassium) are sufficiently soluble to be present almost entirely in the dissolved phase. The concentration of others, in particular calcium phosphate, is higher than can be maintained in solution at the normal pH of milk. Consequently, these exist partly in soluble form and partly in an insoluble or colloidal form associated with casein. The state and distribution of these salts has been extensively reviewed by Pyne (1962) and Holt (1985).

The dividing line between soluble and colloidal is somewhat arbitrary, its exact position depending very much on the method used to achieve separation. However, a fairly sharp separation between the two phases is not difficult since the insoluble salts occur mainly associated with the colloidal casein micelles.

5.7.1 Methods used to separate the colloidal and soluble phases

The methods used include dialysis, ultrafiltration, high-speed centrifugation and rennet coagulation. The method used must not cause changes in equilibrium between the two phases. The two most important precautions are to avoid changes in pH (lowering the pH dissolves colloidal calcium phosphate, see Figure 5.11) and temperature (reducing the temperature dissolves colloidal calcium phosphate and vice versa). Since milk comes from the cow at about 40°C, working at 20°C and especially at 4°C will cause significant shifts in calcium phosphate equilibrium.

Ultrafiltrates obtained using cellophane or polysulphone membranes at 20°C and a transmembrane pressure of *c.* 100 kPa are satisfactory, but the concentrations of citrate and calcium are slightly low due to sieving effects which are accentuated by high pressures. Dialysis of a small volume of water against at least 50 times its volume of milk (to which a little chloroform or azide has been added as preservative) at 20°C for 48 h is the most satisfactory separation procedure and agrees closely with results obtained

Table 5.4 Effect of temperature on the composition of diffusate obtained by dialysis (modified from Davies and White, 1960)

Constituent	$mg\,l^{-1}$ milk	
	20°C	3°C
Total calcium	379	412
Ionized calcium	122	129
Magnesium	78	79
Inorganic phosphorus	318	326
Citrate (as citric acid)	1730	1750
Sodium	580	600
Potassium	1330	1330

Table 5.5 Distribution of salts ($mg\,l^{-1}$ milk) between the soluble and colloidal phases of milk (from Davies and White, 1960)

Constituent	Total in milk	Diffusate	Colloidal
Total calcium	1142	381 (33.5%)	761 (66.5%)
Ionized calcium		117	–
Magnesium	110	74 (67%)	36 (33%)
Sodium	500	460 (92%)	40 (8%)
Potassium	1480	1370 (92%)	110 (8%)
Total phosphorus	848	377 (43%)	471 (57%)
Citrate (as citric acid)	1660	1560 (94%)	100 (6%)
Chloride	1063	1065 (100%)	0 (0%)

by ultrafiltration and renneting techniques, although the latter tends to be slightly high in calcium. As mentioned above, the temperature at which dialysis is performed is important, e.g. diffusate prepared from milk at 3°C contains more total calcium, ionized calcium and phosphate than a diffusate prepared at 20°C (Table 5.4).

The partition of salts between the soluble and colloidal phases is summarized in Table 5.5. In general, most or all of the sodium, potassium, chloride and citrate, one-third of the calcium and two-thirds of the magnesium and about 40% of the inorganic phosphate are in the soluble phase.

The phosphorus of milk occurs in five classes of compounds: phospholipids, lipid, casein, small soluble organic esters, soluble and colloidal inorganic salts (Figure 5.7).

5.7.2 Soluble salts

The soluble salts are present in various ionic forms and unionized complexes. Sodium and potassium are present totally as cations, while chloride

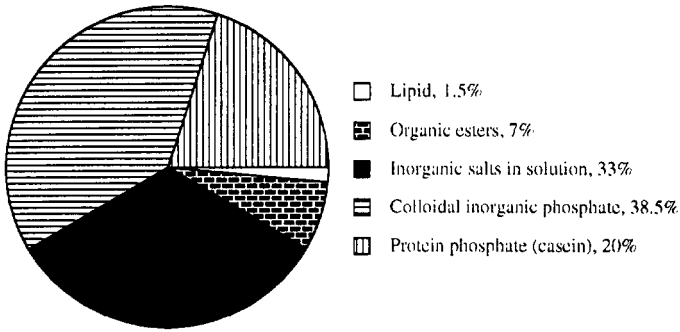


Figure 5.7 Distribution of phosphorus among various classes of compounds in bovine milk.

and sulphate, anions of strong acids, are present as anions at the pH of milk. The salts of weak acids (phosphates, citrates and carbonates) are distributed between various ionic forms, the concentration of which can be calculated approximately from the analytical composition of milk serum and the dissociation constants of phosphoric, citric and carbonic acid, after allowance has been made for binding of calcium and magnesium to citrate as anionic complexes and to phosphate as undissociated salts. The distribution of the various ionic forms can be calculated according to the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\text{salt}]}{[\text{acid}]}$$

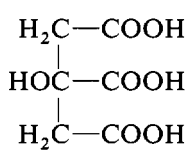
Phosphoric acid (H_3PO_4) dissociates as follows:

$$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-}$$
$$pK_a^1 = 1.96 \qquad pK_a^2 = 6.83 \qquad pK_a^3 = 12.32$$

$H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-} are referred to as primary, secondary and tertiary phosphate, respectively.

The titration curve for H_3PO_4 using NaOH is shown in Figure 5.8.

Citric acid is also triprotic while carbonic acid (H_2CO_3) is diprotic.



Citric acid

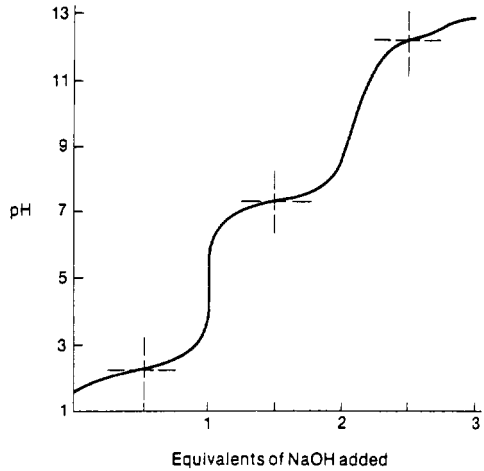


Figure 5.8 Titration curve for phosphoric acid (H_3PO_4); + indicates pK_a^1 (1.96), pK_a^2 (6.8) and pK_a^3 (12.3).

The exact value of the dissociation constants which should be used depends on the total ionic concentration and consequently, the constants used for milk are approximate. The following values are generally used:

Acid	pK_a^1	pK_a^2	pK_a^3
Citric	3.08	4.74	5.4
Phosphoric	1.96	6.83	12.32
Carbonic	6.37	10.25	

In milk, the critical dissociation constants are pK_a^3 for citric acid, pK_a^2 for phosphoric acid and pK_a^1 for carbonic acid. Bearing in mind the limitations and assumptions of the above data, the following calculations can be made for the distribution of the various ions in milk at pH 6.6.

Phosphoric acid. For the first dissociation, $H_3PO_4 \rightleftharpoons H^+ + H_3PO_4^-$; $pK_a^1 = 1.96$

$$pH = pK_a^1 + \log \frac{[salt]}{[acid]}$$

$$6.6 = 1.96 + \log \frac{[salt]}{[acid]}$$

$$\frac{[salt]}{[acid]}, \text{ i.e. } \frac{H_2PO_4^-}{H_3PO_4} = \frac{43\,700}{1}.$$

Therefore, there is essentially no H_3PO_4 in milk.

For the second dissociation, i.e. $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+; pK_a^2 = 6.83$

$$6.6 = 6.83 + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$\log \frac{[\text{salt}]}{[\text{acid}]} = -0.23$$

$$\frac{[\text{salt}]}{[\text{acid}]}, \text{ i.e. } \frac{HPO_4^{2-}}{H_2PO_4^-} = \frac{0.59}{1}.$$

For the third dissociation, i.e. $HPO_4^{2-} \rightleftharpoons PO_4^{3-} + H^+; pK_a^3 = 12.32$

$$6.6 = 12.32 + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$\log \frac{[\text{salt}]}{[\text{acid}]} = -5.72$$

$$\frac{[\text{salt}]}{[\text{acid}]}, \text{ i.e. } \frac{PO_4^{3-}}{HPO_4^{2-}} = \frac{1.9 \times 10^{-6}}{1}.$$

Dihydrogenphosphate (primary) and monohydrogenphosphate (secondary) are the predominant forms, in the ratio of 1.0:0.59, i.e. 63% $H_2PO_4^-$ and 37% HPO_4^{2-}.

Citric acid. Using pK_as of 3.08, 4.74 and 5.4:

$$\frac{H_2\text{Citrate}^-}{H_3\text{Citric acid}} = \frac{3300}{1}$$

$$\frac{H\text{Citrate}^{2-}}{H_2\text{Citrate}^-} = \frac{72}{1}$$

$$\frac{\text{Citrate}^{3-}}{H\text{Citrate}^{2-}} = \frac{16}{1}$$

Therefore, tertiary (Citrate^{3-}) and secondary ($H\text{Citrate}^{2-}$) citrate, in the ratio 16:1, are the predominant forms.

Carbonic acid. The small amount of carbonic acid present occurs mainly as the bicarbonate anion, HCO_3^-.

Calcium and magnesium. Some calcium and magnesium in milk exist as complex undissociated ions with citrate, phosphate and bicarboante, e.g. Ca Citr^-, $CaPO_4^-$, Ca HCO_3^+. Calculations by Smeets (1955) suggest the following distribution for the various ionic forms in the *soluble* phase:

- Calcium + magnesium: 35% as ions, 55% bound to citrate and 10% bound to phosphate.

Table 5.6 Distribution of milk salts

Species	Concentration ($mg\,l^{-1}$)	Soluble		Colloidal (%)
		%	form	
Sodium	500	92	Completely ionized	8
Potassium	1450	92	Completely ionized	8
Chloride	1200	100	Completely ionized	–
Sulphate	100	100	Completely ionized	–
Phosphate	750	43	10% bound to Ca and Mg 51% H_2PO^- 39% HPO_4^{2-}	57
Citrate	1750	94	85% bound to Ca and Mg 14% $Citrate^{3-}$ 1% $HCitrate^{2-}$	
Calcium	1200	34	35% Ca^{2+} 55% bound to citrate 10% bound to phosphate	66
Magnesium	130	67	Probably similar to calcium	33

- Citrates: 14% tertiary ($Citrate^{3-}$), 1% secondary ($HCitrate^{2-}$) and 85% bound to calcium and magnesium.
- Phosphates: 51% primary ($H_2PO_4^-$), 39% secondary (HPO_4^{2-}) and 10% bound to calcium and magnesium.

Combining this information with the distribution of the various salts between the colloidal and soluble phases (Table 5.5), gives the quantitative distribution of the salts in milk shown in Table 5.6.

It should be possible to determine experimentally the concentrations of anions such as HPO_4^{2-} and $Citrate^{3-}$ in milk using ion-exchange resins or by nuclear magnetic resonance spectroscopy, but no such experimental work has been reported and available data are by calculation only.

Making certain assumptions and approximations as to the state of various ionic species in milk, Lyster (1981) and Holt, Dalgleish and Jenness (1981) developed computer programs that permit calculation of the concentrations of various ions and soluble complexes in typical milk diffusate. The outcome of both sets of calculations are in fairly good agreement and are also in good agreement with the experimentally determined values for those species for which data are available. The values calculated by Holt, Dalgleish and Jenness (1981) are shown in Table 5.7.

The ionic strength of milk is around 0.08 M.

5.7.3 Measurement of calcium and magnesium ions

Ca^{2+} and Mg^{2+}, along with H^+, play especially important roles in the stability of the caseinate system and its behaviour during milk processing, especially in the coagulation of milk by rennet, heat and ethanol. The

Table 5.7 Calculated concentrations (mM) of ions and complexes in a typical milk diffusate (from Holt, Dalgleish and Jenness, 1981)

Anion	Free ion	Cation complex			
		Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}
H_2Cit^-	+	+	+	+	+
$HCit^{2-}$	0.04	0.01	+	+	+
Cit^{3-}	0.26	6.96	2.02	0.03	0.04
$H_2PO_4^-$	7.50	0.07	0.04	0.10	0.18
HPO_4^{2-}	2.65	0.59	0.34	0.39	0.52
PO_4^{3-}	+	0.01	+	+	+
GLC-1-HPO_4^-	0.50	+	+	0.01	0.01
GLC-1-PO_4^{2-}	1.59	0.17	0.07	0.10	0.14
H_2CO_3	0.11	–	–	–	–
HCO_3^-	0.32	0.01	+	+	+
CO_3^{2-}	+	+	+	+	+
Cl^-	30.90	0.26	0.07	0.39	0.68
HSO_4^-	+	+	+	+	+
SO_4^{2-}	0.96	0.07	0.03	0.04	0.10
RCOOH	0.02	–	–	–	–
$RCOO^-$	2.98	0.03	0.02	0.02	0.04
Free ion	–	2.00	0.81	20.92	36.29

+, <0.005 μM; –, not estimated; GLC, glucose.

concentrations of these ions are also related to the solubility of the colloidal calcium phosphate. Consequently, there is considerable interest in determining their concentrations; three methods are available:

Cation-exchange resins. Using ion-exchange resins, Ca^{2+} and Mg^{2+} are adsorbed on to a cation-exchange resin added to milk; the resin is removed and the Ca^{2+} and Mg^{2+} desorbed. It is assumed that the treatment does not alter the ionic equilibrium in milk.

Interaction with murexide. The murexide method depends on the formation of a complex between Ca^{2+} and ammonium purpurate (murexide, M):

$$Ca^{2+} + M \rightleftharpoons Ca\,M$$

The free dye (M) has an absorption maximum at 520 nm while Ca M absorbs maximally at 480 nm. The concentration of Ca^{2+} can be calculated from a standard curve in which A_{480} is plotted as a function of $[Ca^{2+}]$ or preferably from a standard curve of $(A_{520} - A_{480})$ as a function of $[Ca^{2+}]$ which is less curved and more sensitive (Figure 5.9). Using this method, the $[Ca^{2+}]$ in milk was found to be 2.53–3.4 mM and appears to be 0.8 mM higher than that determined by the other methods.

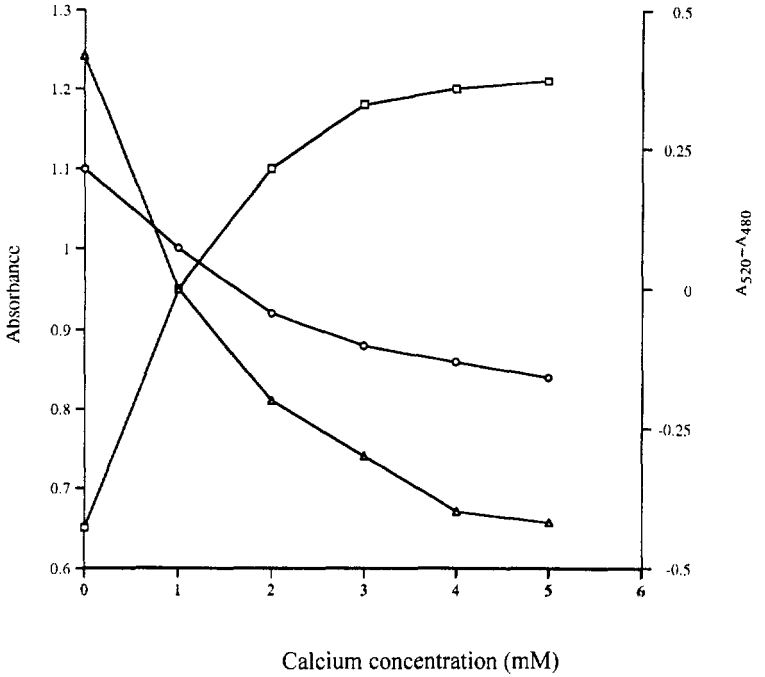


Figure 5.9 Standard curve for the absorbance of murexide at 520 nm (○) and of Ca-murexide at 480 nm (□) and $A_{520} - A_{480}$ (△).

The murexide method measures Ca^{2+} only; Mg^{2+}, at the concentration in milk, does not affect the indicator appreciably. Calculation of Mg^{2+} concentration is possible when the total calcium and magnesium (obtained by EDTA titration) is known. This is based on the assumption that the same proportion of each cation is present in the ionic form, which is justifiable since the dissociation constants of their citrate and phosphate salts are virtually identical.

Ca-ion electrode. Ca^{2+} activity (rather than concentration) can be determined rapidly and accurately using a Ca^{2+} ion-specific electrode. Care must be exercised to ensure that the potentiometer is properly standardized using solutions that simulate the composition of milk serum. The Ca^{2+} activity is lower than the Ca^{2+} concentration – values of about 2 mM have been reported.

5.7.4 Colloidal milk salts

As shown in Table 5.5, all the major ionic species in milk, with the exception of Cl^-, are distributed between the soluble and colloidal phases, but the

principal colloidal salt is calcium phosphate; about 67% and 57%, respectively, of the total calcium and phosphate are in the colloidal phase. The colloidal inorganic salts are, therefore, frequently referred to as **colloidal calcium phosphate** (CCP), although some sodium, potassium, magnesium, zinc and citrate are also present in the colloidal phase. CCP is closely associated with the casein micelles and there are two principal questions as to its nature:

- its composition and structure;
- the nature of its association with casein.

Composition and structure. All the colloidal sodium ($40\,mg\,l^{-1}$), potassium ($110\,mg\,l^{-1}$) and most of the magnesium ($30\,mg\,l^{-1}$) are probably associated with the casein as counter-ions to the negatively charged organic phosphate and carboxylic acid groups of the protein. It has been calculated that approximately 30% of the colloidal calcium (*c.* $250\,mg\,l^{-1}$) is also directly attached to these groups. According to most authors (Pyne, 1962), casein is capable of binding 25–30 moles calcium per 10^5 g casein (i.e. about 1160 g calcium per 10^5 g casein). Assuming that milk contains 25 g casein l^{-1}, the calcium-binding potential of the casein is about $300\,mg\,l^{-1}$ of milk. Since the neutralizing potential of Na^+ and K^+ is half that of Ca^{2+} and Mg^{2+}, the binding capacity of $300\,mg\,l^{-1}$ is reasonably close to the sum of the values given above.

These calculations leave about 500 mg of calcium and about 350 mg of phosphate present in the colloidal phase per litre of milk to be accounted for. The available evidence suggests that the excess CCP is present largely as tricalcium phosphate, $Ca_3(PO_4)_2$, or some similar salt.

The so-called Ling oxalate titration indicates that CCP consists of 80% $Ca_3(PO_4)_2$ and 20% $CaHPO_4$, with an overall Ca:P ratio of 1.4:1 (Pyne, 1962). However, the oxalate titration procedure has been criticized because many of the assumptions made are not reliable. Pyne and McGann (1960) developed a new technique to study the composition of CCP. Milk was acidified to about pH 4.9 at 2°C, followed by exhaustive dialysis of the acidified milk against a large excess of bulk milk; this procedure restored the acidified milk to normality in all respects except that CCP was not reformed. Analysis of milk and CCP-free milk (assumed to differ from milk only in respect of CCP) showed that the ratio of Ca:P in CCP was 1.7:1. The difference between this value and that obtained by the oxalate titration (i.e. 1.4:1) was attributed to the presence of citrate in the CCP complex, which is not measured by the oxalate method. Pyne and McGann (1960) suggested that CCP has an apatite structure with the formula:

$$3Ca_3(PO_4)_2, CaHCitr^- \quad \text{or} \quad 2.5Ca_3(PO_4)_2, CaHPO_4, 0.5\,Ca_3Citr_2^-.$$

Based on the assumption that the amount of Ca bound directly to casein is equivalent to the number of ester phosphate groups present, Schmidt

(1982) argues that CCP is most likely to be amorphous tricalcium phosphate [$Ca_3(PO_4)_2$]. The argument is as follows: It is likely that the phosphoserine residues of the caseins are potential sites for interaction with CCP. The importance of these residues in calcium binding has been demonstrated also for dentine and salivary phosphoproteins. In a casein micelle of particle weight 10^8 Da, consisting of 93.3% casein, with an ester phosphorus content of 0.83%, there are 25 000 ester phosphate groups. Such a micelle contains about 70 500 calcium atoms and about 30 000 inorganic phosphate residues, from which 5000 $Ca_9(PO_4)_6$ clusters might be formed, leaving 25 500 calcium atoms. This means that there is aproximately one calcium atom for each ester phosphate group and that about 40% of these ester phosphate groups can be linked in pairs via $Ca_9(PO_4)_6$ clusters, as shown in Figure 5.10. It is suggested that $Ca_9(PO_4)_6$ clusters adsorb two calcium atoms, which easily fit into the crystal grid, and thus acquire a positive charge and can interact electrostatically with the negatively charged ester phosphate groups of casein. The proposed structure and association with the casein micelles is shown in Figure 5.10.

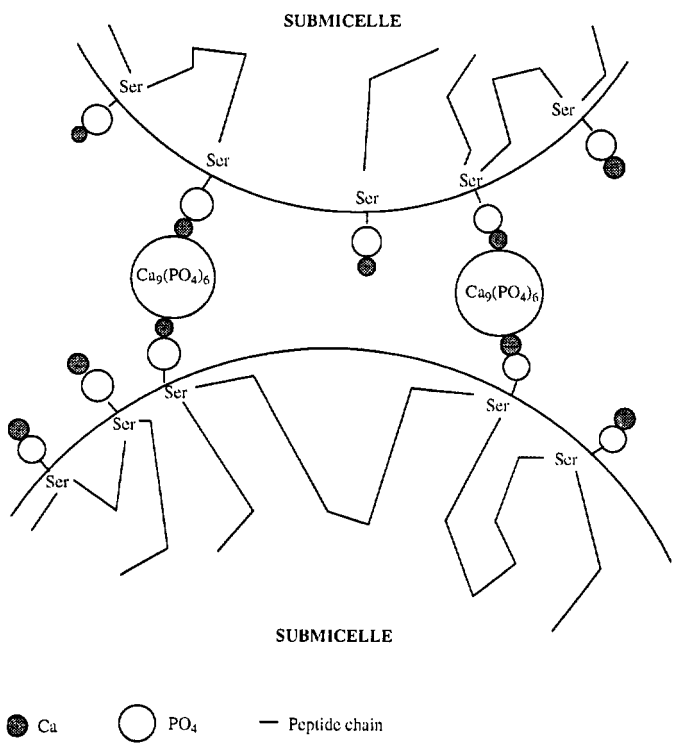


Figure 5.10 Association of colloidal calcium phosphate ($Ca_3(PO_4)_2$) with the serine phosphate groups of casein (from Schmidt, 1982).

The best physical studies, using various forms of X-ray spectroscopy, on the structure of CCP have been undertaken by Holt and colleagues (Holt, 1985). It was concluded that the most likely form of CCP is brushite ($CaHPO_4.2H_2O$), which has also been identified in bone and other calcified tissues. He explains the difference between the Ca/P ratio found by analysis, i.e. 1.51–1.6 and the Ca/P ratio of $CaHPO_4$, i.e. 1.0, as being due to the ability of the phosphate moiety of phosphoserine to substitute in surface sites of a brushite-type lattice.

Association with casein. The colloidal calcium phosphate is closely associated with the casein; it does not precipitate out of solution and is considered to be protected against precipitation by the casein. Two possible forms of protection are suggested:

- physical protection;
- chemical association between CCP and casein.

Experimental evidence strongly favours the idea of chemical association:

- CCP remains attached to the casein following treatment with protein dissociating agents (e.g. urea) or following proteolysis.
- Comparison of the potentiometric titration curves of milk and CCP-free milk shows more reactive organic phosphate groups in the latter, suggesting that CCP is attached to the organic casein phosphate groups, thereby rendering them less active.
- The formol titration is not influenced by removal of CCP, suggesting that εNH_2-groups of lysine are not involved.

The views of Schmidt and Holt on the association between CCP and casein, i.e. via a shared Ca^{2+} (Schmidt) or a shared phosphoserine, i.e. phosphoserine as part of the CCP crystal lattice (Holt), support the hypothesis of chemical association.

Although CCP represents only about 6% of the dry weight of the casein micelle, it plays an essential role in its structure and properties and hence has major effects on the properties of milk; it is the integrating factor in the casein micelle; without it, milk is not coagulable by rennet and its heat and calcium stability properties are significantly altered. In fact, milk would be a totally different fluid without colloidal calcium phosphate.

As discussed in Chapter 4 (p. 186), Holt (1994) has proposed that casein has evolved with the ability to bind high concentrations of calcium and phosphate so that milk can contain high levels of these ions, which are essential for neonatal growth, without precipitation in the ducts of the mammary glands.

5.8 Changes in milk salts equilibria induced by various treatments

The equilibria between the soluble and colloidal salts of milk are influenced by many factors, the more important of which are discussed below, and which consequently modify the processing properties of milk.

Milk serum is supersaturated with calcium phosphate, the excess being present in the colloidal phase, as described above. The balance between the colloidal and soluble phases may be upset by various factors, including changes in temperature, dilution or concentration, addition of acid, alkali or salts. The solubility product for secondary calcium phosphate, $[Ca^{2+}][HPO_4^{2-}]$ is about 1.5×10^{-5} or $pK_s = 4.85$.

5.8.1 Addition of acid or alkali

Acidification of milk is accompanied by a progressive solubilization of colloidal calcium phosphate and other colloidal salts from casein. Solubilization is complete below about pH 4.9 (Figure 5.11).

Addition of alkali has the opposite effect, and at about pH 11 almost all the soluble calcium phosphate occurs in the colloidal phase. These changes are not reversible on subsequent dialysis against untreated milk.

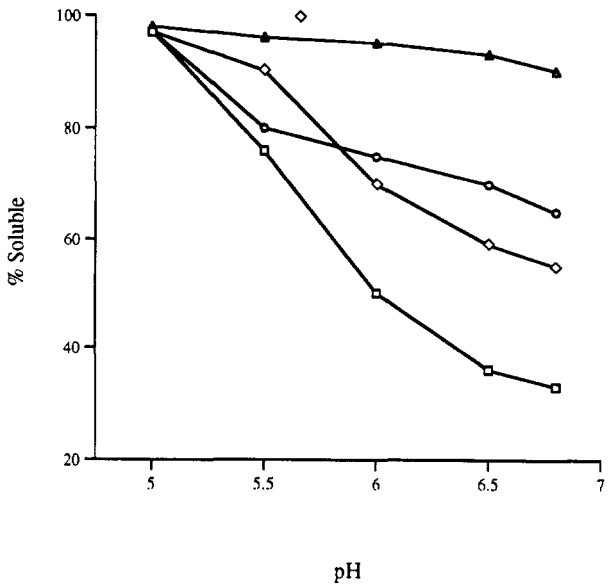


Figure 5.11 Effect of pH on the distribution of calcium (□), inorganic phosphorus (◇), magnesium (○) and citrate (△) between the colloidal and soluble phases in bovine milk.

5.8.2 *Addition of various salts*

Divalent cations. Addition of calcium to milk causes precipitation of soluble phosphate as colloidal calcium phosphate, an increase in ionized calcium, a decrease in the concentration of soluble phosphate and a decrease in pH.

Phosphate. Addition of secondary Na or K phosphate (i.e. Na_2HPO_4 or K_2HPO_4) causes the precipitation of colloidal calcium phosphate, with concomitant decreases in the concentration of soluble calcium and calcium ion. Polyphosphates, e.g. Na-hexametaphosphate, chelate Ca^{2+} strongly and dissolve CCP.

Citrate. Addition of citrate reduces the concentrations of calcium ions and colloidal calcium phosphate and increases the soluble calcium, soluble phosphate and pH.

5.8.3 *Effect of changes in temperature*

The solubility of calcium phosphate is markedly temperature-dependent. Unlike most compounds, the solubility of calcium phosphate decreases with increasing temperature; therefore, heating causes precipitation of calcium phosphate while cooling increases the concentrations of soluble calcium and

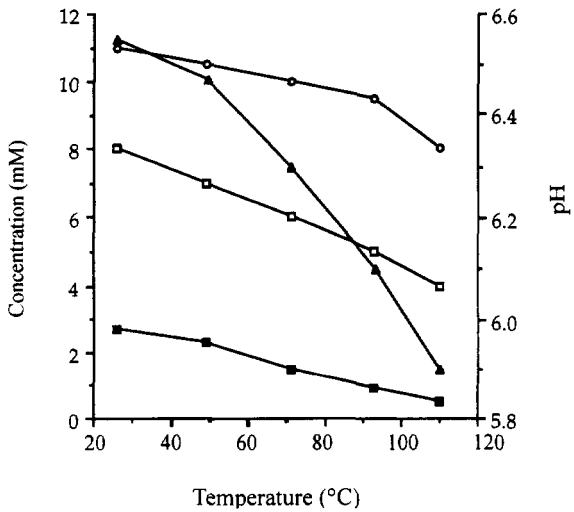


Figure 5.12 Concentration of total calcium (□), calcium ions (■), phosphate (○) and pH (▲) of ultrafiltrates prepared from milk at various temperatures (from Rose and Tessier, 1959).

phosphate at the expense of CCP. At low temperatures, shifts in the ionic balance are readily reversible, but after heating at high temperatures, reversibility becomes more sluggish and incomplete. Comparatively slight changes (20 to 3°C) cause substantial changes in equilibrium (Table 5.4) which are completely reversible. The effects of high temperature treatments were studied by Rose and Tessier (1959) using ultrafiltration of milk at various temperatures. Calcium and phosphate precipitate rapidly on heating (essentially complete within 5 min), to an extent dependent on temperature (Figure 5.12), but the distribution of Na, K, Mg or citrate are not affected. On cooling, these changes are partly reversible.

5.8.4. *Changes in pH induced by temperature*

The pH of milk is changed following heating due to changes in two salt systems. Fresh milk contains 200 mg $CO_2\,l^{-1}$; about 50% of this is lost on standing, with additional losses on heating. This results in a decrease in titratable acidity and an increase in pH. The formation of colloidal calcium phosphate during heating more than compensates for the loss of CO_2. The effect of temperature on pH is shown in Table 5.8 and Figure 5.12.

The change in pH can be described as follows:

$$3Ca^{2+} + 2HPO_4^{2-} \underset{\text{cooling}}{\overset{\text{heating}}{\rightleftharpoons}} Ca_3(PO_4)_2 + 2H^+$$

The reaction is reversible on cooling after heating to moderate temperatures but becomes only partially reversible following more severe heating. The shifts in calcium phosphate equilibrium and pH increase when milk is concentrated.

5.8.5 *Effect of dilution and concentration*

Since milk is saturated with respect to calcium and phosphate, dilution reduces the concentration of Ca^{2+} and HPO_4^{2-} and causes solution of some colloidal calcium phosphate, making the milk more alkaline. Concentration

Table 5.8 Effect of temperature on the pH of milk

Temperature (°C)	pH
20	6.64
30	6.55
40	6.45
50	6.34
60	6.23

of milk causes precipitation of colloidal phosphate and shifts the reaction of milk to the acid side, e.g. concentration by a factor of 2:1 reduces the pH to 6.2.

$$\text{Dilution:} \quad Ca_3(PO_4)_2 \xrightarrow{H_2O} 3Ca^{2+} + 2HPO_4^{2-} + 2OH^-$$

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

5.8.6 Effect of freezing

Freezing milk causes crystallization of pure water and the unfrozen liquid becomes more saturated with respect to various salts. Some soluble calcium phosphate precipitates as $Ca_3(PO_4)_2$, with the release of H^+ and a decrease in pH (e.g. to 5.8 at $-20°C$).

As discussed in Chapter 2 (p. 38), crystallization of lactose as α-monohydrate exacerbates the situation. The combination of increased concentrations of Ca^{2+} and reduced pH causes destabilization of the casein micelles.

References

Davies, D.T. and White, J.C.D. (1960) The use of ultrafiltration and dialysis in isolating the aqueous phase of milk and in determining the partition of milk constituents between the aqueous and disperse phases. *J. Dairy Res.*, **27**, 171–90.

Flynn, A. and Power, P. (1985) Nutritional aspects of minerals in bovine and human milks, in *Developments in Dairy Chemistry*, Vol. 3: *Lactose and Minor Constituents* (ed. P.F. Fox), Elsevier Applied Science, London, pp. 183–215.

Holt, C. (1981) Some principles determining salt composition and partitioning of ions in milk. *J. Dairy Sci.*, **64**, 1958–64.

Holt, C. (1985) The milk salts: their secretion, concentrations and physical chemistry, in *Developments in Dairy Chemistry*, Vol. 3: *Lactose and Minor Constituents* (ed. P.F. Fox), Elsevier Applied Science, London, pp. 143–81.

Holt, C. (1994) The biological function of casein, in *Yearbook 1994, The Hannah Research Institute*, Ayr, Scotland, pp. 60–8.

Holt, C., Dalgleish, D.G. and Jenness, R. (1981) Calculation of the ion equilibria in milk diffusate and comparison with experiment. *Anal. Biochem.*, **113**, 154–63.

Jenness, R. (1988) Composition of milk, in *Fundamentals of Dairy Chemistry*, 3rd edn (ed. N.P. Wong), Van Norstrand Reinhold, New York, pp. 1–38.

Lyster, R.L.J. (1981) Calculation by computer of individual concentrations in simulated milk salt solution. II. An extension to the previous model. *J. Dairy Res.*, **48**, 85–9.

Pyne, G.T. (1962) Some aspects of the physical chemistry of the salts of milk. *J. Dairy Res.*, **29**, 101–30.

Pyne, G.T. and McGann T.C.A. (1960) The colloidal phosphate of milk. II. Influence of citrate. *J. Dairy Res.*, **27**, 9–17.

Rose, D. and Tessier, H. (1959) Composition of ultrafiltrates from milk heated at 80 to 230°F in relation to heat stability. *J. Dairy Sci.*, **42**, 969–80.

Schmidt, D.G. (1982) Association of caseins and casein micelle structure, in *Developments in Dairy Chemistry*, Vol. 1: *Proteins* (ed. P.F. Fox), Applied Science, London, pp. 61–86.

Smeets, W.J.G.M. (1955) The determination of the concentration of calcium ions in milk ultrafiltrate. *Neth. Milk Dairy J.*, **9**, 249–60.

Suggested reading

Flynn, A. and Cashman, K. (1997) Nutritional aspects of minerals in bovine and human milks, in *Advanced Dairy Chemistry*, Vol. 3: *Lactose, Water, Salts and Vitamins*, 2nd edn (ed. P.F. Fox), Chapman & Hall, London, pp. 257–302.

Holt, C. (1985) The milk salts: their secretion, concentrations and physical chemistry, in *Developments in Dairy Chemistry*, Vol. 3: *Lactose and Minor Constituents* (ed. P.F. Fox), Elsevier Applied Science, London, pp. 143–81.

Holt, C. (1997) The milk salts and their interaction with casein, in *Advanced Dairy Chemistry*, Vol. 3, *Lactose, Water, Salts and Vitamins*, 2nd edn (ed. P.F. Fox), Chapman & Hall, London, pp. 233–56.

Jenness, R. and Patton, S. (1959) *Principles of Dairy Chemistry*, New York, John Wiley Sons.

Jensen, R.G. (ed.) (1995) *Handbook of Milk Composition*, Academic Press, San Diego.

Pyne, G.T. (1962) Some aspects of the physical chemistry of the salts of milk. *J. Dairy Res.*, **29**, 101–30.