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ARTICLE

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An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk

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Abstract An equilibrium thermodynamic model of the interaction of calcium, phosphate and casein in milk is described in which the micellar calcium phosphate is assumed to be in the form of calcium phosphate nanoclusters. A generalized empirical formula for the nanocluster is used to define the molar ratios of small ions (Ca, Mg, P_i and citrate) to a casein phosphorylated sequence (phosphate centre, PC). From this model, a method of calculating the partition of milk salts into diffusible and non-diffusible fractions is obtained. No arbitrary assumptions are made, no fitting of adjustable parameters is done and the PCs in the caseins are defined by inspection of their primary structures. In addition to the salt partition, the mole fractions of the individual caseins not complexed to the calcium phosphate through one or more of their PCs are computed and a generic stability rule for milks is derived. The use of the model is illustrated by calculations of the partition of salts in a standard milk and by comparison with experimental data on the partition of salts in the milk of individual cows. The generic stability rule is applied to the individual milks to determine whether the micellar calcium phosphate is thermodynamically stable. According to the calculations, compositions that might lead to pathological calcification in the lumen of the mammary gland were seldom found in primiparous healthy cows in early or mid lactation but occurred more often in multiparous animals, in late lactation and during mastitic infection.

Keywords Calcification · Calcium phosphate · Casein · Milk · Salt partition

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Abbreviations ACP: amorphous calcium phosphate · Cit: citrate · CN: casein · CPN: calcium phosphate nanocluster · DCPD: dicalcium phosphate dihydrate · HA: hydroxyapatite · IAP: ion activity product · MCP: micellar calcium phosphate · MWCO: molecular weight cut-off · OCP: octacalcium phosphate · PC: phosphate centre · TCC: tricalcium citrate

Introduction

Scientific studies of the partition of the salts in cows' milk (for reviews, see Pyne 1934, 1962; Holt 1997) have shown that about a third of the calcium, half the P_i, two thirds of the Mg and 90% of the citrate (Cit) are diffusible through a semi-permeable membrane with a molecular weight cut-off (MWCO) of about 10 kDa (Davies and White 1960). Very similar results can be obtained by partitioning the milk salts by ultrafiltration of milk at low pressure through a membrane of suitable porosity (Davies and White 1960). The great majority of the non-diffusible salts are in the form of colloidal calcium phosphate or, preferably, micellar calcium phosphate (MCP).

The salt (White and Davies 1958; McGann and Pyne 1960) and phosphopeptide (Holt et al. 1986; Ono et al. 1994) composition of MCP has been determined approximately from the non-diffusible concentrations of ions or by isolation of calcium phosphate-rich particles from proteolytic digests of casein micelles. A precise composition has been difficult to obtain because some of the non-diffusible ions may not be part of the calcium phosphate and the extent of involvement of the casein phosphate groups is not easy to determine experimentally. The location of the non-diffusible Mg and Cit is not yet established to be exclusively in the MCP. With these uncertainties, quite different views have been expressed of the nature of the calcium phosphate based on analytical results such as the non-diffusible Ca/P_i ratio

or the ingenious oxalate titration method of Pyne and Ryan (1932) for determining the acidity of the salt. The Ca/P_i ratio of the non-diffusible salts is about 2, which favours a basic salt if only the inorganic salts are relevant (Pyne and Ryan 1932; McGann and Pyne 1960; Pyne and McGann 1960; McGann et al. 1983a, 1983b), but the ratio $Ca/(P_i + P_o)$ is almost equal to that of a dicalcium phosphate when all the casein phosphates (P_o) are added (Holt et al. 1982, 1989).

A wide range of structural methods has been deployed to determine the structure of MCP, including electron microscopy (Knoop et al. 1979; McGann et al. 1983a, 1983b), electron diffraction in combination with high-resolution electron microscopy (Lyster et al. 1984), X-ray absorption spectroscopy near the K-absorption edge of calcium, infrared spectroscopy (Holt et al. 1982; Holt and Hukins 1991) and solid-state ^{31}P nuclear magnetic resonance spectroscopy (Thomsen et al. 1995; Ailamo et al. 1996; Rasmussen et al. 1997; Bak et al. 2001). It has been established beyond reasonable doubt that MCP particles are several nanometres in size, that they are formed from an amorphous hydrated calcium phosphate linked to casein phosphate centres (PCs) and that they are distributed throughout the protein matrix of casein micelles. Holt and co-workers (Holt et al. 1986; Holt and Hukins 1991) hypothesized on the basis of structural, solubility, titration and compositional results that the MCP particles are essentially nuclei of an amorphous dicalcium phosphate phase containing all the casein phosphate groups. That is, that they are in a metastable state where growth into a macroscopic phase has been arrested by the inhibitory effect of the casein. Schmidt (1982), van Dijk (1990a, 1990b, 1991) and van Dijk and Hersevoort (1992) have put forward other models for MCP with just a few calcium and phosphate ions in a cluster, but they do not account for the electron-dense, nanometre-sized, regions of micelles seen by electron microscopy and other structural methods (Knoop et al. 1979; Holt and Hukins 1991; McMahon and McManus 1998) and it is doubtful, on general chemical grounds, whether such small clusters could have anything more than a transient existence. Solid-state ^{31}P NMR spectroscopy has shown (Bak et al. 2001) at least three components in the casein micelle spectrum obtained by slow-speed spinning single-pulse or cross-polarization magic angle spinning methods. The two major components, of approximately equal importance, have the broad bandwidth expected of an amorphous solid and were identified on the basis of their isotropic and anisotropic chemical shifts with inorganic phosphate (PO_4^{3-}) and casein phosphate. The third peak, with a narrower bandwidth, was considered to be in rapid exchange with the continuous phase and amounted to about 8% of the total. The dynamic nature of the ion cluster has also been studied by solution NMR methods, including use of the stable quadrupolar ^{43}Ca and ^{25}Mg nuclei (Wahlgren et al. 1990), and chemical exchange methods with radioisotopes (Yamauchi et al. 1969; Yamauchi and Yoneda 1977; Pierre et al. 1983; Zhang and Aoki 1996). There are very slow (hours or days), slow (minutes), intermediate (seconds) and fast (milliseconds or faster) rates of exchange of the non-diffusible inorganic constituents. In general, all the ions are exchangeable within an hour or so at elevated temperature (Pierre et al. 1983). In a recent study, Kolar et al. (2002) subjected their $^{32}P_i$ exchange results to a compartment analysis and observed three compartments with residence times at room temperature of 818, 0.24 and 23 h.

Calcium phosphate nanoclusters (CPNs) can be prepared by pH adjustment of a solution containing certain casein phosphopeptides and milk salts (Holt et al. 1996, 1998; Holt 2001; Little and Holt 2004). However, they can also be formed by spontaneous re-dispersion of a fresh precipitate of amorphous calcium phosphate (ACP), at least in the presence of citrate (Holt 2001; Little and Holt 2004). These and other studies have shown that the CPN is an equilibrium cluster of defined composition in which a core of acidic ACP is surrounded by a shell of the phosphopeptide, with the phosphorylated residues forming the interface. Unlike MCP, the composition of CPN can be established precisely because they are formed from solutions of a simple and fully specified composition. A chemical thermodynamic model has been used successfully to calculate the extent of reaction using an invariant ion activity product (IAP) and a defined chemical composition for the complex with the phosphopeptide (Little and Holt 2004).

The proposition that MCP and CPN are essentially the same (Holt et al. 1996) is supported by a number of observations. Thus, they have comparable quantitative compositions, containing an ACP and casein PCs, and the CPN may also incorporate Mg and Cit if it is prepared from a solution containing these ions. Opinions differ on how acidic is the ACP in MCP (Holt 1982; Chaplin 1984; Bak et al. 2001), but the invariant IAP for milk and that for CPN are quite similar (Holt 1982; Little and Holt 2004) and of the form expected for an acidic calcium phosphate. The size of the electron dense regions in the micelle is comparable to the size of the core of the CPN (Knoop et al. 1979; McGann et al. 1983b; McMahon and McManus 1998). Furthermore, if it is assumed that the mass of the calcium phosphate in the MCP is the same as the core mass of the CPN, then the neutron scattering behaviour of the casein micelle can be explained (Holt et al. 2003). In particular, the well-established substructure correlation length of 18 nm (Stothart and Cebula 1982), which various authors have attributed to protein subunits (Stothart 1989; Hansen et al. 1996; Walstra 1999), is explained as an interparticle interference effect of the CPN-like particles within the micelle (Holt et al. 2003). According to the CPN substructure model, there are about 800 such calcium phosphate particles in a casein micelle of radius 100 nm. Since the α_{s1}- and α_{s2}-caseins contain more than one PC per molecule, and hence can, in principle, bridge between different calcium phosphate cores, and since

there are about 50 PCs per shell, there is considerable scope for cross-linking a substantial proportion of caseins in the micelle.

In this paper, it is shown that the method of calculating the extent of reaction in forming the CPN (Little and Holt 2004) can be adapted and applied to milk. No arbitrary assumptions are made, no fitting of adjustable parameters is done and the PCs are defined by inspection of the primary structures of the caseins. The procedure has been used to calculate the partition of salts and the composition of an equilibrium milk ultrafiltrate as a function of pH in a standard milk. In addition to the salt partition, the mole fractions of the individual caseins not complexed to calcium phosphate may also be computed and a generic stability rule for milk has been derived. Results are compared to experimental values of the natural variations in salt partition in cows' milk using the well-regarded data of White and Davies (1958), incorporating subsequent corrections for the Cit concentrations (White and Davies 1963) and amalgamation with the casein composition data of Davies and Law (1977). No previous theoretical model of the partition of salts in milk has, to the author's knowledge, been reported.

Materials and methods

Theory

Figure 1 depicts three alternative routes for the spontaneous change of a supersaturated solution into various products, depending on the phosphopeptide concentration. Although dicalcium phosphate dihydrate (DCPD) can nucleate directly from solution, under many conditions of supersaturation and pH, an initial metastable ACP is formed first (Fig. 1A) and this undergoes Ostwald ripening into a more crystalline state such as hydroxyapatite (HA). Although low concentrations of phosphopeptides may delay nucleation and growth from moderately or highly supersaturated solutions, once precipitation begins, it will usually overwhelm the capacity to form nanoclusters. At higher phosphopeptide concentrations, no precipitate or colloidal particles appear because of the preferential formation of the CPN (Fig. 1C).

Thermodynamic model of the standard milk

The standard milk composition approximates that found in bulk milks of Ayrshire cows (White and Davies 1958; Davies and Law 1977) and to that degree can be regarded as representative of the milk of many other western breeds. It differs from real milk in having no γ-casein, which is considered to be a breakdown product of β-casein by the natural milk proteinase, plasmin, and the very many minor anions of milk are gathered together into the two groups of carboxylic acids (RCOOH) and ester phosphates (Glc-1-P). The total concentrations of the standard milk are given in Table 1.

Also shown in Table 1 are the names of the 19 Gibbs components that are used in the thermodynamic description of the milk. Convenient choices for the Gibbs components are electroneutral species such as the bases NaOH, $Ca(OH)_2$, etc., and the acids, HCl, H_3PO_4, etc., or the analytical values, Ca, Mg, etc. The proteins are in a hypothetical electroneutral state where all the acidic residues and the C-terminus are protonated and the N-terminus and all the basic residues (K, R and H) are unprotonated. It is important to recognize that a Gibbs component such as $Ca(OH)_2$ is the sum of all chemical species containing calcium, one of which is the chemical species $Ca(OH)_2$, though at milk pH this particular species is present at only a very low concentration. In milk there is a continuous phase and a fat phase. The proteins are treated as part of the continuous phase in the thermodynamic model. The calculation of the chemical species from the model allows the further calculation of the species able to permeate a dialysis or ultrafiltration membrane of a given porosity and this provides a ready means of testing the model against experiment.

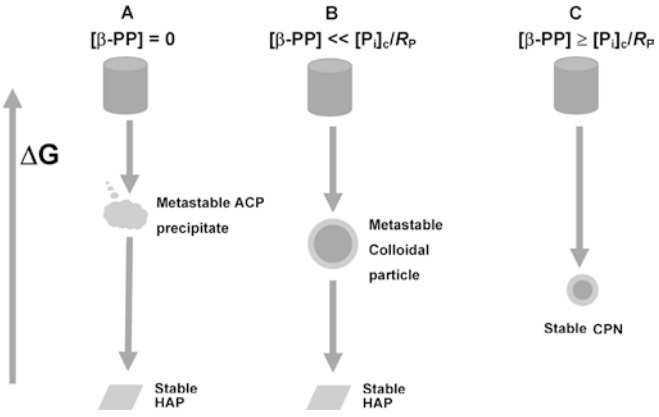


Fig. 1 Depiction of the result of precipitating calcium phosphate from a supersaturated solution when it contains (**A**) no β-PP, (**B**) sufficient β-PP to form metastable colloidal particles, and (**C**) excess β-PP over that which is required to form calcium phosphate nanoclusters

Table 1 Salt and casein composition[a] (mM), fat, whey protein lactose and water concentrations (g L^{-1}) and pH of a standard cows' milk (White and Davies 1958) and its equilibrium diffusate, with corrected citrate values (White and Davies 1963) and individual casein concentrations[a] (Davies and Law 1977)

Constituent	Total	Diffusate	Gibbs component	Constituent	Total
Ca	29.0	10.2	$Ca(OH)_2$	κ-Casein	0.170
Mg	4.9	3.4	$Mg(OH)_2$	β-Casein	0.458
Na	22.0	22.0	NaOH	α_{s1}-Casein	0.419
K	38.3	38.0	KOH	α_{s2}-Casein	0.104
P_i	20.6	12.4	H_3PO_4	Fat	37
Cit	9.5	9.4	H_3Cit	Lactose	46
Cl	30.4	32.3	HCl	Whey protein	6
Ester P	2.6	2.6	H_2RPO_4	Water	880
Carbonate	0.4	0.4	H_2CO_3	pH	6.70
Sulfate	1.2	1.2	H_2SO_4		
Misc. acids	3.1	3.1	RCOOH		

[a]Recalculated from the weight fractions of Davies and Law (1977) using the mole fraction data given in Table 6

Calculation of the diffusible ion concentrations in milk from the composition of an equilibrium ultrafiltrate

Table 1 gives the total concentrations of the standard ultrafiltrate prepared from the standard milk and this can be used to calculate the composition of the diffusible ions in the continuous phase of the milk. The ionic species in the ultrafiltrate may be calculated from the Gibbs components, as previously described (Holt et al. 1981), and the results are shown in Table 2.

Each diffusible chemical species is in electrochemical equilibrium across the semi-permeable membrane. That is, the chemical potential of the diffusible species i in the milk, $\mu_{m,i}$, is equal to its chemical potential in the ultrafiltrate, $\mu_{u,i}$. Expanding the chemical potentials in the usual way gives:

$$\mu_{u,i} = \mu_i^\circ + RT\ln\{X_i\}_u + Z_i F\psi_u$$
$$\mu_{m,i} = \mu_i^\circ + RT\ln\{X_i\}_d + Z_i F\psi_m \quad (1)$$

where R is the gas constant, T the temperature in K, F the Faraday constant, $\{X_i\}$ the activity of X_i and ψ the electrical potential. Collecting terms gives:

$$\{X_i\}_d/\{X_i\}_u = \exp\left[\frac{Z_i F\Delta\psi}{RT}\right] \quad (2)$$

where $\Delta\psi = \psi_u - \psi_m$. In an equilibrium ultrafiltrate at 25 °C, $\Delta\psi \approx 10$ mV, corresponding roughly with the value expected of a Donnan potential arising from the net negative charge on the non-diffusible proteins. The effect is to generate a pH difference of about 0.02 units between milk and the ultrafiltrate, but care is needed in such measurements because of the equilibrium with CO_2 and the unusually large temperature dependence of milk pH (Chaplin and Lyster 1988).

To convert the activities to concentrations requires an activity coefficient. This is made up of a Debye–Hückel screening of the coulomb potential giving rise to an activity coefficient γ and an effect of the excluded volume of co-solutes, ϕ. The γ term is calculated from Debye–Hückel theory, or a modification taking account of the finite size of the ions, or read from tables of activity coefficients measured in simple electrolyte solutions. The overall activity coefficient, Γ, is then $\Gamma = \gamma/(1-\phi)$. Equation (2) becomes:

$$[X_i]_d/[X_i]_u = \left(\frac{\gamma_{u,i}(1-\phi_m)}{\gamma_{d,i}(1-\phi_m)}\right)\exp\left[\frac{Z_i F\Delta\psi}{RT}\right] \quad (3)$$

The contributions to the excluded volume fraction, ϕ_m, arising from typical milk values of 4% fat, 3.4% protein and 4.6% lactose (Table 1) are approximately 0.044, 0.024 and 0.030, respectively, whereas only the lactose value contributes to ϕ_u. Since the γ terms are likely to be very nearly equal, the first term in parentheses in Eq. (3) is $0.902/0.970 = 0.930$, whereas for skim milk the ratio is 0.98. For cationic species the Donnan and excluded volume terms in Eq. (3) tend to cancel out, but for anionic species they are both less than unity.

Calculation of the diffusible concentrations in milk is made by applying Eq. (3) to each of the species in Table 2 and summing the rows and columns. For the Gibbs component $[Ca(OH)_2]_d$, the summation is:

$$[Ca(OH)_2]_d = [Ca^{2+}]_d + [CaCit^-]_d + [CaHPO_4]_d + [CaHCit]_d + [CaH_2PO_4^+]_d + \ldots \quad (4)$$

The non-diffusible concentration of a component is *defined* as the difference between the total and diffusible concentrations in the milk; for example:

$$[Ca(OH)_2]_c = [Ca(OH)_2]_t - [Ca(OH)_2]_d \quad (5)$$

An example calculation of the diffusible and non-diffusible salt concentrations is given in Table 3 using the total and ultrafiltrate compositions and ion species in the ultrafiltrate, as given in Tables 1 and 2.

Table 2 Concentrations (mM) of the free ions and complexes calculated from the standard diffusate composition given in Table 3 (Holt et al. 1981)

Anion	Free ion	Complex Ca^{2+}	Mg^{2+}	Na^+	K^+
H_2Cit^-	+[a]	+	+	+	+
$HCit^{2-}$	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-PH^-	0.50	+	+	0.01	0.01
Glc 1-P^{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^-	30.9	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

[a]Concentrations shown as (+), <0.005 mM; (–), not determined

Calculation of the partition of milk salts in a CPN solution

CPNs have been prepared from the individual casein (CN) phosphopeptides β-CN 4P (f1–25), β-CN 5P (f1–41), α_{s1}-CN 5P (f59–79) and from a mixture of casein phosphopeptides derived from a tryptic or pronase digestion of whole casein (Holt et al. 1996, 1998; Holt 2001; Little and Holt 2004). Most work has been done with the β-CN 4P (f1–25) peptide, which has yielded an empirical formula for the CPN of:

$$Ca_{R_{Ca}}Mg_{R_{Mg}}(Cit)_{R_{Cit}}(P_i)_{R_P}\beta\text{–CN 4P (f1 – 25)} \quad (6)$$

In a milk-like solution of salts, $R_{Ca} = 13.2$, $R_{Mg} = 1.0$, $R_{Cit} = 1.3$ and $R_P = 6.5$ (Little and Holt 2004). The extent of reaction of the phosphopeptide in forming the CPN is estimated and the ion equilibria among the remaining ions and free peptide are computed. The appropriate IAP is calculated and compared to the equilibrium value. An adjustment is made to the extent of reaction and the cycle repeated until equilibrium is attained. From the chemical species present at equilibrium, it is possible to compute the diffusible concentrations and compare the predicted composition of an equilibrium ultrafiltrate with experiment. To make a similar calculation for milk requires that the chemical composition of the MCP be defined in terms of PCs.

Casein phosphate centres

The term PC is defined here as being formed from a nucleus of at least two phosphorylated residues in a short sequence, including the phosphokinase recognition site. In Table 4, the mole fractions of the four bovine caseins are calculated and the positions of the PCs in the sequences of the secreted polypeptide chains are identified. If all the caseins have their maximum number, then κ-, β-, α_{s1}- and α_{s2}-caseins have 0, 1, 2 and 3 PCs, respectively. It must be recognized that there is a possibility for PCs to be enlarged or even created by higher levels of structure than the primary one, as is indeed the case in the formation of many binding sites and active sites in globular proteins. All of the caseins contain some isolated phosphorylated residues that might augment the nucleus of a PC in the casein micelle.

Table 3 Calculated diffusible and non-diffusible concentrations in the standard milk using the composition and species distribution given in Tables 1 and 2

Component	Total	Diffusible	Non-diffusible
Ca	29.0	9.6	19.4
Mg	4.9	3.2	1.7
Na	22.0	21.0	1.0
K	38.3	36.2	2.2
P_i	20.6	11.4	9.2
Cit	9.5	8.8	0.7
Cl	30.4	30.4	0.0

Table 4 Davies and Law (1977) data on bovine casein mole fractions and identification of candidate PCs from the primary structure only

Casein	Mole fraction	Maximum no. of PCs	Phosphoamino acids (max.)	Candidate PCs
κ	0.13	0	1 (3)	None
β	0.41	1	4	f15–21
α_{s1}	0.37	2	2 (3)	f41–50
			4	f64–70
α_{s2}	0.09	3	3	f8–12
			4	f56–63
			2 (3)	f129–133

If a PC is required to contain a minimum number, s, of phosphorylated residues, then the maximum number of PCs is the total number of phosphorylated residues divided by s. For $s=4$, the smallest number of phosphorylated residues in a short peptide that has so far been found to be capable of forming nanoclusters, the calculation of the number of potential PCs in the caseins is again 0, 1, 2 and 3 PCs in κ-, β-, α_{s1}- and α_{s2}-caseins, respectively. The number average of PCs per polypeptide chain in the standard milk is then 1.42.

There is some experimental evidence that, after tryptic digestion, the more highly phosphorylated peptides remain bound to the MCP, but peptides with 0, 1 or 2 phosphorylated residues are not retained or are only poorly retained in the isolated MCP (Holt et al. 1986; Aoki et al. 1992; Ono et al. 1994). Nevertheless, the 3P or even the 2P PCs in the intact protein may be bound to the calcium phosphate in the native micelle to form the nucleus from which a larger PC forms and, as discussed above, any PCs that are assembled from two or more otherwise remote parts of the sequence may dissociate after proteolysis.

Calculation of the partition of caseins

Many manipulations of milk result in a change of pH or other ion concentrations having some consequence for the organization of caseins into micelles. The micelles are not completely cross-linked by bridging chains of the α_s-caseins, so the response to environmental perturbation can be a change in the size distribution of the micelles as well as dissociation of individual caseins or small molecular weight particles from micelles. In principle, the partition of caseins into diffusible and non-diffusible fractions can be accomplished through a membrane of suitable porosity and compared with theory.

The caseins exist in a variety of different genetic, glycosylated and phosphorylated forms (Table 4). Let the mole fractions of all forms with 0, 1, 2, 3, ... PCs be x_0, x_1, x_2 and x_3, ... x_{max}, respectively. For example, all the forms of κ-casein may comprise x_0, β-casein may comprise x_1, α_{s1}-casein B 8P and 9P may comprise x_2 and the various phosphorylated forms of α_{s2}-casein may comprise x_3 ($=x_{max}$).

Casein of type i, mole fraction x_i and number of PCs i, can exist either free of any links to the calcium phosphate (x_i^{f}) or be bound to the calcium phosphate (x_i^{b}). All the bound fraction is in micelles, whereas the free fraction may be further partitioned into a fraction that is potentially diffusible through a membrane of suitable porosity ($x_i^{f,d}$) and a fraction that is bound to the micelle through interactions with proteins ($x_i^{f,m}$):

$$\begin{aligned} x_i &= x_i^{f} + x_i^{b} \\ x_i^{f} &= x_i^{f,d} + x_i^{f,m} \end{aligned} \tag{7}$$

If all the PCs are independent and of equal reactivity, the distribution of species is given by:

$$x_{i,j} = \alpha^j (1-\alpha)^{i-j} \frac{i!}{(i-j)!j!} x_i \tag{8}$$

where casein of type i and mole fraction x_i with i PCs has j reacted PCs. Hence, the total free concentration, i.e. $j=0$, is:

$$x_i^{f} = x_i^{f,d} + x_i^{f,m} = (1-\alpha)^i x_i \tag{9}$$

Let K_i be the partition coefficient for casein type i between the continuous phase and the micelle when it is not bound to the calcium phosphate of the micelle:

$$K_i = \frac{x_i^{f,d}}{x_i^{f,m}} \tag{10}$$

The use of a simple partition coefficient cannot be fully justified since the caseins will aggregate to form a variety of states other than casein micelles and single molecules. It is used here as a simple introduction to how the casein partition may be calculated. Combining Eqs. (9) and (10) and rearranging gives the fraction of free casein of type i in the continuous phase, θ_i, to be:

$$\theta_i = \frac{x_i^{f,d}}{x_i} = \left(\frac{K_i}{1+K_i}\right)(1-\alpha)^i \tag{11}$$

Using Eq. (3) for the mole fractions of caseins with no reacted PCs allows the reacted fraction to be computed, giving:

$$\bar{i}^{b} = \frac{\sum_i [1-(1-\alpha)^i] i x_i}{\sum_i [1-(1-\alpha)^i] x_i} \tag{12}$$

where the summations extend over all i, $0 \le i \le i_{max}$. Equation (12) has the limiting forms:

$$\begin{aligned} \bar{i}^{b}_{\alpha\to 0} &= \frac{\sum_{i=1}^{i_{max}} i^2 x_i}{\sum_{i=1}^{i_{max}} i x_i} \\ \bar{i}^{b}_{\alpha\to 1} &= \frac{\sum_{i=1}^{i_{max}} i x_i}{\sum_{i=1}^{i_{max}} x_i} \end{aligned} \tag{13}$$

so that for the casein mole fractions in Table 4, $1.63 \le \bar{i}^{b} \le 1.90$ (Fig. 2a).

The concentration of reacted PCs is obtained by summation of the terms in Eq. (8):

$$\alpha \sum_i i x_i = \alpha \bar{i} = \sum_{i=1}^{i_{max}} \sum_{j=1}^{i} \alpha^j (1-\alpha)^{i-j} \frac{i!}{(i-j)!(j-1)!} x_i \tag{14}$$

where:

$$\bar{i} = \frac{\sum_i i x_i}{\sum_i x_i} \tag{15}$$

Figure 2 shows how $\bar{i}^{b}$ and the x_i^{b} vary with α and how, as a result, the composition of the bound casein becomes progressively richer in the two α_s-caseins as the extent of reaction is decreased.

Empirical formula of MCP and calculation of the non-diffusible concentrations of salts

The generalization of Eq. (6) for a mixture of different and partly reacted caseins is:

$$Ca_{R_{Ca}} Mg_{R_{Mg}} (Cit)_{R_{Cit}} (P_i)_{R_P} (PC)_1 (CN)_{1/\bar{j}} \tag{16}$$

where the number average of reacted PCs per bound casein molecule is

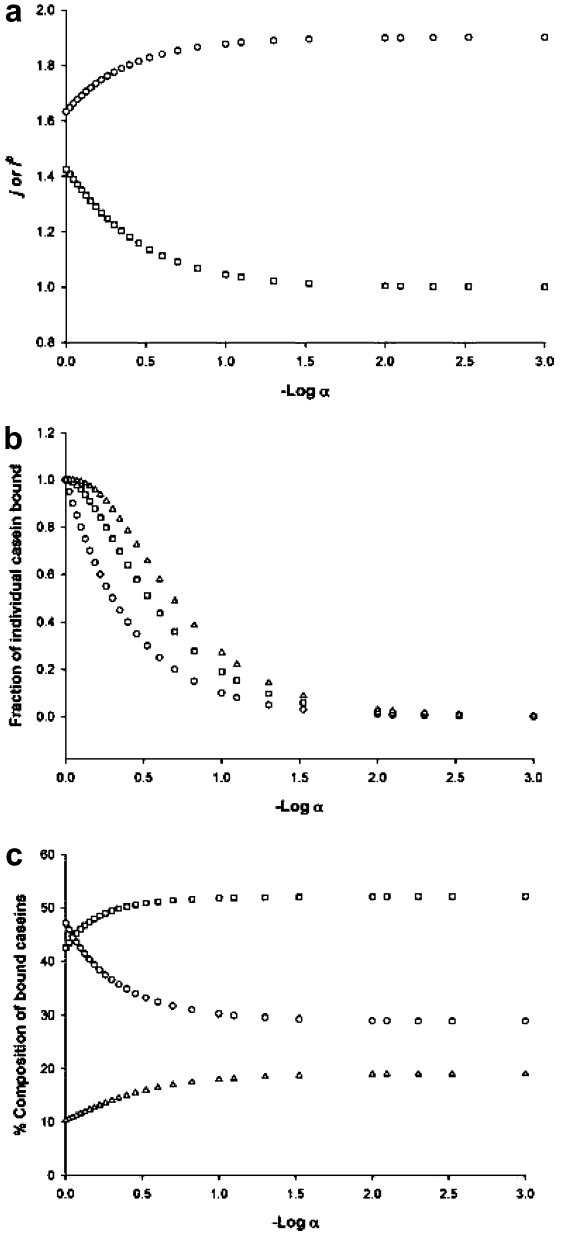


Fig. 2a, b Calculated mole fractions of individual caseins complexed in the form of MCP. (**a**) The average number of phosphate centres per casein polypeptide chain bound to the calcium phosphate, $\bar{i}^{b}$ (*circles*), or the average number of reacted phosphate centres per bound casein polypeptide chain, $\bar{j}$ (*squares*). (**b**) Mole fractions of β- (*circles*), α_{s1}- (*squares*) and α_{s2}-caseins (*triangles*) bound to the calcium phosphate particles versus $-\log\ \alpha$. (**c**) Percentage composition of the caseins bound to calcium phosphate as a function of the extent of reaction (same symbols as **b**)

$$\bar{j} = \frac{\sum_{i=1}^{i_{max}} \sum_{j=1}^{i} j x_{i,j}}{\sum_{i=1}^{i_{max}} \sum_{j=1}^{i} x_{i,j}} = \frac{\sum_{i=1}^{i_{max}} \sum_{j=1}^{i} \frac{i!}{(i-j)!(j-1)!} \alpha^{j} (1-\alpha)^{i-j} x_i}{\sum_{i=1}^{i_{max}} \sum_{j=1}^{i} \frac{i!}{(i-j)!j!} \alpha^{j} (1-\alpha)^{i-j} x_i} \tag{17}$$

Thus the MCP is regarded as having a fixed composition, apart from the number of polypeptide chains. The variation of $\bar{j}$ with α is shown in Fig. 2a. In the limit as α tends to zero and unity, $\bar{j}$ tends to 1 and $\bar{i}$, respectively.

There are essentially only two contributions to the non-diffusible salt concentrations. These are non-diffusible salts comprising the MCP and other cations bound to the unreacted PCs. Both of these terms can be expressed as functions of α. The unbound PCs are assumed to act as binding sites for small ions in competitive equilibria to give, principally, the isotherm functions $\bar{\nu}_{Ca}$, $\bar{\nu}_{Mg}$, and $\bar{\nu}_{H}$ in units of mol ion per mol PC. It is assumed, as before (Little and Holt 2004), that the Ca and Mg ions compete for the same binding sites with the same association constants and that the ratio of the concentrations of bound ligands is therefore proportional, at a given pH, to the ratio of the free ion concentrations. It is further assumed that the binding properties of whole casein are the sum of the independent and equal binding properties of the unreacted PCs. The total non-diffusible concentrations of Ca, Mg and H are then given by:

$$[\mathrm{Ca}]_c = \bar{i}[\mathrm{CN}](R_{Ca}\alpha + (1-\alpha)\bar{\nu}_{Ca}(\mathrm{pH}, \{Ca^{2+}\}, \{Mg^{2+}\})) \tag{18}$$

$$[\mathrm{Mg}]_c = \bar{i}[\mathrm{CN}](R_{Mg}\alpha + (1-\alpha)\bar{\nu}_{Mg}(\mathrm{pH}, \{Ca^{2+}\}, \{Mg^{2+}\})) \tag{19}$$

$$[\mathrm{H}]_c = \bar{i}[\mathrm{CN}](R_{H}\alpha + (1-\alpha)\bar{\nu}_{H}(\mathrm{pH}, \{Ca^{2+}\}, \{Mg^{2+}\})) \tag{20}$$

For the anions Cit and P_i, only a single term is needed since they are not thought to bind to caseins other than through the MCP:

$$[\mathrm{Cit}]_c = \bar{i}[\mathrm{CN}]R_{Cit}\alpha \tag{21}$$

$$[\mathrm{P_i}]_c = [\mathrm{P_i^b}] = \bar{i}[\mathrm{CN}]R_{P}\alpha \tag{22}$$

It follows from Eqs. (18)–(22) that in the limit $\alpha \to 1$, all the non-diffusible ion concentrations are in constant proportion, in accordance with the fixed composition of the MCP.

Thermodynamic stability of milk

In the model, MCP is in the thermodynamically stable form of a CPN. In some milks, however, there may not be enough PCs to sequester all the calcium phosphate in the stable state of nanoclusters and such milks are liable to give rise to calcium phosphate deposits outside the casein micelle. Since $\bar{i}[\mathrm{CN}]$ is the total concentration of PCs and $[\mathrm{P_i^b}]/R_P$ is the concentration of reacted PCs:

$$\alpha = \frac{[\mathrm{P_i^b}]}{R_P \bar{i}[\mathrm{CN}]} \tag{23}$$

from which the following necessary (but not sufficient) condition for thermodynamic stability of the milk is derived:

$$[\mathrm{P_i^b}] \leqslant R_P \bar{i}[\mathrm{CN}] \tag{24}$$

since $0 \leq \alpha \leq 1$. Alternatively, if all the phosphorylated residues are able to contribute to the PCs, the condition of stability can be expressed as:

$$\frac{[\mathrm{P_i^b}]}{[\mathrm{P_o}]} \leqslant R_P \tag{25}$$

Ion activity products in milk and calculation of the chemical species

In natural variations among milk ultrafiltrates or ultrafiltrates from the cow, woman and goat, an invariant IAP was obtained for an empirical formula close to that of a dicalcium phosphate. For example, for the cow the formula is $Ca(HPO_4)_{0.7}(PO_4)_{0.2}$ (Holt 1982) and:

$$-\log_{10} K_{S,\mathrm{MCP}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}^{0.7}\{PO_4^{3-}\}^{0.2}\right) = 6.80 \tag{26}$$

Chaplin (1984) varied the pH in cows' milk by addition of base or acid and obtained an invariant IAP of:

$$-\log_{10} K_{S,\mathrm{MCP}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}\right) = 6.6 \tag{27}$$

For natural variations in the milk of sows in early lactation, the relationship is very similar to that in the cow (Kent et al. 1998):

$$-\log_{10}K_{S,\mathrm{MCP}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}^{0.65}\{PO_4^{3-}\}^{0.23}\right) \quad (28)$$

whereas a slightly more acidic formula was obtained by analysis of goats' milk (Holt 1993):

$$-\log_{10}K_{S,\mathrm{MCP}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}\right) = 6.78 \quad (29)$$

and women's milk (Holt 1993):

$$-\log_{10}K_{S,\mathrm{MCP}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}\right) = 6.51 \quad (30)$$

In studies of CPN formation, Little and Holt (2003) found an invariant IAP with β-CN 4P (f1–25) in a solution that contained neither Mg nor Cit:

$$-\log_{10}K_{S,\mathrm{CPN}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}^{0.4}\{PO_4^{3-}\}^{0.4}\right) = 7.8 \quad (31)$$

In a buffer containing a milk-like salt composition, including Mg and Cit:

$$-\log_{10}K_{S,\mathrm{CPN}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}^{0.4}\{PO_4^{3-}\}^{0.4}\right) = 8.0 \quad (32)$$

and for the commercial phosphopeptide mixture in a milk-like buffer, including Mg and Cit:

$$-\log_{10}K_{S,\mathrm{CPN}} = -\log_{10}\left(\{Ca^{2+}\}\{HPO_4^{2-}\}^{0.4}\{PO_4^{3-}\}^{0.4}\right) = 7.9 \quad (33)$$

Although the K_S values are not strictly comparable because of dimensional differences and in the milk experiments there is an accumulation of experimental errors in the large number of chemical analyses, it is nevertheless clear that fairly consistent y and K_S values are obtained in milk and highly consistent y and K_S values are obtained in nanocluster solutions, but for reasons that are not yet clear the core of the calcium phosphate in MCP is more acidic and soluble than the core of the CPN prepared in the laboratory. Until this issue is resolved, it is necessary to use the pair of constants applicable to the particular system.

The calculation of the species present in cows' milk follows closely that used for CPN solutions (Little and Holt 2004). A value is chosen for α and the chemical species present are calculated using the chemical formula for the MCP (Eq. 16). The ion equilibria are computed to see if the IAP is equal to the appropriate K_S; if it is not, an adjustment is made to α and the cycle repeated until the condition of equilibrium is met. The prediction of the salt partition does not employ any arbitrary assumptions. All of the model parameter values are experimentally derived from independent experiments and the number of casein PCs is justified by inspection of the primary structures.

Table 3 gives the diffusible concentrations calculated at the natural pH of the standard milk and Fig. 3 shows the calculated non-diffusible concentrations as a function of pH. The point of discontinuity near pH 5.8 in many of the lines in Fig. 3 corresponds to the onset of formation of the MCP. The number of PCs was 0, 1, 2 and 3 for κ-, β-, α_{s1}- and α_{s2}-caseins, respectively, and these allowed the nanocluster complexes to form preferentially in the range $5.8 \le \mathrm{pH} \le 7.0$, as shown in Fig. 3a.

Figure 3e shows that the continuous phase is supersaturated with respect to DCPD. Other calculations (Table 5) show that it is supersaturated also with respect to octacalcium phosphate (OCP) and HA over the pH range 5–7, but the basic ACP of Mayer and Eanes (1978) is not predicted to form. Since OCP and HA invariably form through Ostwald ripening of an initial basic ACP, these crystalline phases cannot form. Additionally, caseins can act as powerful inhibitors of crystal growth of DCPD, OCP and HA near neutral pH (van Kemenade and de Bruyn 1989a, 1989b). Below pH 6.3, the continuous phase is supersaturated with respect to tricalcium citrate (TCC) and this phase is the only one that could form, at room temperature, below pH 5. It has been observed to form in some heated, acidified, wheys. The non-diffusible concentrations of Ca and P_i vary with pH, as shown in Fig. 3d, and, over the range where the nanoclusters are present, the $[Ca]_c$ are nearly linearly related to $[P_i]_c$ with a slope close to 2 and a finite intercept (Fig. 3f), as observed experimentally (Holt 1982; Dalgleish and Law 1989).

Consistency of the thermodynamic and structural models of the casein micelle

The casein micelle has been described as having a more-or-less uniform protein matrix in which calcium phosphate nanocluster-like particles are dispersed (Holt et al. 2003). The scattering of X-rays and neutrons by the micelles can show a subsidiary maximum or shoulder corresponding to a substructure correlation length, λ, of about 18 nm and this has been explained as arising from the interference of radiation scattered by the array of nanoclusters within the micelle. In a micelle of mass M and intrinsic viscosity $[\eta]$ containing a mass fraction w of calcium phosphate, in the form of nanoclusters with a core mass M_{core}, the number of nanoclusters is wM/M_{core} and hence:

$$\lambda^3 \approx \frac{2M_{\mathrm{core}}[\eta]}{5wN_{\mathrm{A}}} \quad (34)$$

A nearly equivalent argument uses the number of reacted PCs, $\alpha\bar{i}M/M_{\mathrm{o}}$, where M_{o} is the number average mass of the caseins, giving $\alpha\bar{i}M/M_{\mathrm{o}}n_{\mathrm{PC}}$ for the number of nanoclusters, where n_{PC} is the number of PCs in a nanocluster. Hence:

$$\alpha = \frac{n_{\mathrm{PC}}wM_{\mathrm{o}}}{\bar{i}M_{\mathrm{core}}} \quad (35)$$

which may be compared to Eq. (23). The correlation length can then be expressed in an alternative way as:

$$\lambda^3 \approx \frac{2n_{\mathrm{PC}}M_{\mathrm{o}}[\eta]}{5\alpha\bar{i}N_{\mathrm{A}}} \quad (36)$$

Taking typical values for micelles of $M_{\mathrm{o}} = 23$ kDa, $w = 0.07$, $\bar{i} = 1.42$ and $[\eta] = 10$ mL g^{-1} (De Kruif and Holt 2003), together with the properties of the CPN, $M_{\mathrm{core}} = 61$ kDa and $n_{\mathrm{PC}} = 49$, gives $\lambda = 18.0$ nm from Eq. (34), $\alpha = 0.91$ from Eq. (35) and $\lambda = 18.0$ nm from Eq. (36). Thus the thermodynamic and structural models appear to be consistent with one another.

Results and discussion

Partition of milk salts

The compositional data of White and Davies (1958) for Ayrshire cows are among the most complete analyses reported for the milk of any species. The analyses were extensive, covering 15, 55 and 15 samples of milk from individual cows in early, middle and late lactation, respectively, together with 22 samples from cows with mastitis. The survey was comprehensive, in that the concentrations of nearly all the components needed for the thermodynamic model were measured, with the principal exception that only the total casein concentration was determined. Analyses were also reported for low-pressure ultrafiltrates of the milks, which allows a comparison to be made between calculations of the ultrafiltrate concentrations from the model and the

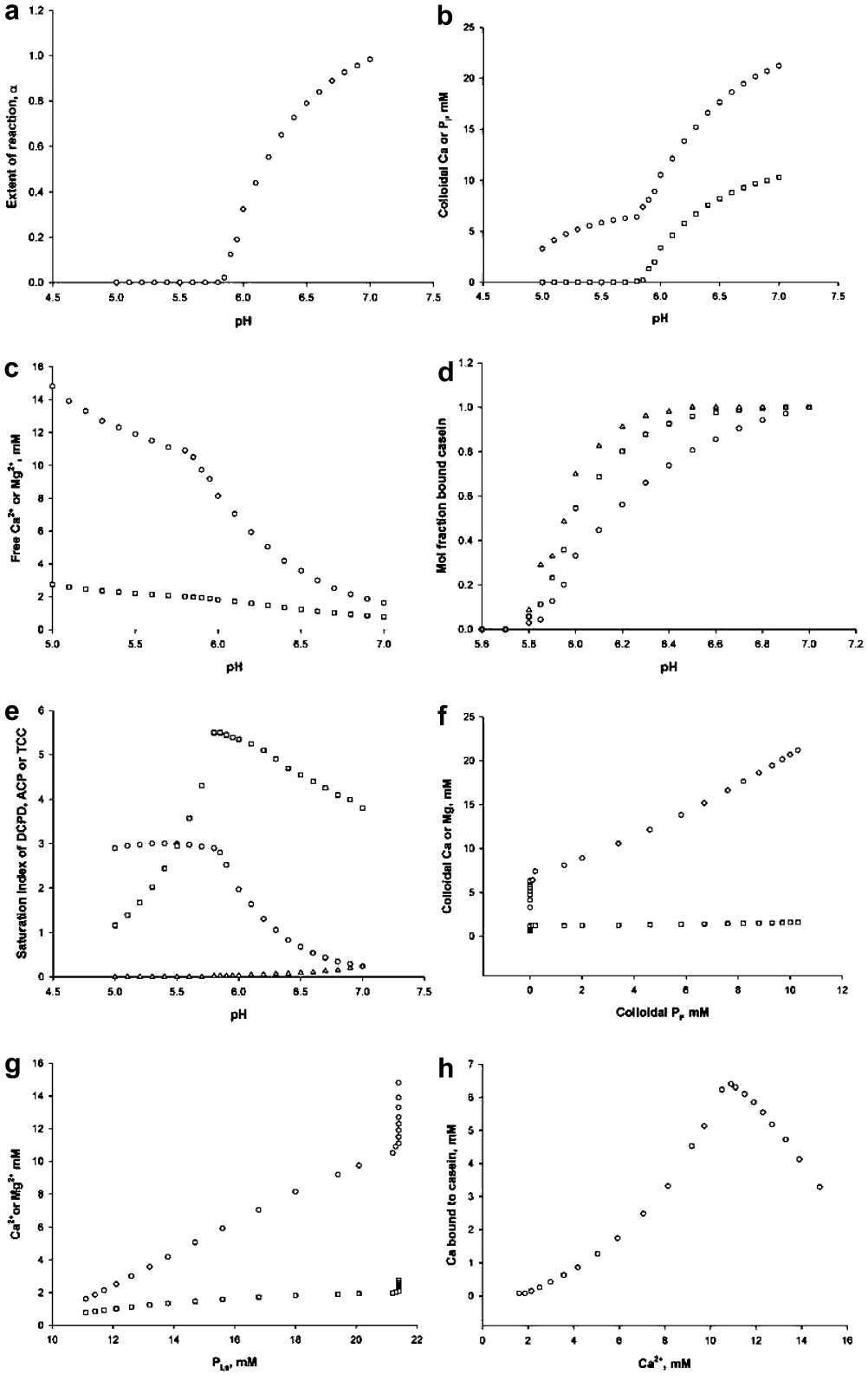


Fig. 3a–h Partition of salts in the model skim milk of Tables 1 and 3 calculated using Eqs. (18)–(22), with Eq. (26) used to define the IAP and Eq. (16) to define the molar ratios in the MCP. The casein phosphate centres per mole were $\kappa = 0$, $\beta = 1$, $\alpha_{s1} = 2$ and $\alpha_{s2} = 3$. (**a**) Extent of reaction versus pH. (**b**) Non-diffusible Ca (*circles*) and P_i (*squares*) versus pH. (**c**) Free Ca^{2+} (*circles*) and Mg^{2+} (*squares*) versus pH. (**d**) Mole fractions of β- (*circles*), α_{s1}- (*squares*) and α_{s2}-caseins (*triangles*) bound to the calcium phosphate particles versus pH. (**e**) Saturation indices for TCC (*circles*), DCPD (*squares*) and ACP (*triangles*) versus pH. (**f**) Non-diffusible Ca (*circles*) and Mg (*squares*) versus non-diffusible P_i. (**g**) Free Ca^{2+} (*circles*) and Mg^{2+} (*squares*) versus diffusible P_i. (**h**) Casein-bound Ca versus free Ca^{2+}

ultrafiltration results (Davies and White 1960). Subsequently, Davies and Law (1977) determined the weight concentrations of the α_{s1}-, α_{s2}-, β-, γ- and κ-caseins in a different group of Ayrshire cows in early, middle and late lactation. These two data sets were combined to provide all the values needed for the thermodynamic model.

The casein weight concentrations were converted to molar concentrations using the molecular mass and microvariant composition figures given in Table 6

Table 5 Chemical formulae and solubility products of some pure calcium phosphates and other potential solid phases in the standard milk. The saturation indices (SI = IAP/solubility product) were calculated from the diffusible ion activities given in Table 1

Compound	Abbreviation	Formula	Ca/P	$-\log_{10}$ (IAP)	SI
Dicalcium phosphate	DCP	$CaHPO_4$	1.0	6.90	3
Dicalcium phosphate dihydrate	DCPD	$CaHPO_4.2H_2O$	1.0	6.59	6
Micellar calcium phosphate hydrate	MCP	$Ca(HPO_4)_{0.7}(PO_4)_{0.2}.xH_2O$	1.1	6.80	1
Octacalcium phosphate	OCP	$Ca_8H_2(PO_4)_6.5H_2O$	1.33	96.6	60
β-Tricalcium phosphate	β-TCP	β-$Ca_3(PO_4)_2$	1.5	28.9	200
Hydroxyapatite	HA	$Ca_5OH(PO_4)_3$	1.67	58.4	8×10^8
Amorphous calcium phosphate	ACP	$Ca_3(HPO_4)_{0.2}(PO_4)_{1.87}.xH_2O$	1.45	24.8	0.2
Tricalcium citrate dihydrate	TCC	$Ca_3(Cit)_2.2H_2O$	–	17.6	0.4
Dimagnesium phosphate	–	$MgHPO_4$	–	5.82	0.3

Table 6 Average mole fractions of individual caseins in the milk of Ayrshire cows in early, middle and late lactation. Calculated from the original data of Davies and Law (1977)

Casein	Genetic variant	Secondary modification	Mass (Da)	Average mole fraction (SD)		
				Early	Middle	Late
α_{s1}	100% B	8P:9P = 9:1	23,623	0.373 (0.010)	0.378 (0.016)	0.358 (0.022)
α_{s2}	100% A	10P:11P:12P:13P = 1:4:4:1	25,270	0.098 (0.023)	0.081 (0.026)	0.072 (0.019)
β	100% A	100% 5P	23,983	0.306 (0.031)	0.355 (0.022)	0.305 (0.037)
γ_1	100% A	100% 1P	20,520	0.013 (0.005)	0.014 (0.007)	0.025 (0.009)
γ_2	100% A	100% 0P	11,821	0.039 (0.022)	0.025 (0.019)	0.059 (0.031)
κ	50% A 50% B	40% glycosylation	19,280	0.149 (0.011)	0.147 (0.017)	0.181 (0.025)

and the averages were used to calculate $\bar{t}$. A similar calculation was made for the bulk milk samples of Davies and Law (1977), yielding $\bar{t} = 1.42 \pm 0.04$. For early, middle and late lactation milks the results were 1.40 ± 0.05, 1.38 ± 0.07 and 1.32 ± 0.06, respectively. In making this calculation it was assumed that the β-casein contribution of PCs is the sum of the mole fractions of the extant protein and the γ-caseins, on the grounds that the latter are breakdown products of the action of the milk proteinase plasmin on the β-casein (reviewed by Fox 1992) and that the complementary phosphopeptides are retained in the milk. The slightly smaller value for $\bar{t}$ in late lactation is due to two factors, namely an increase in κ-casein and a decrease in α_{s2}-casein, of which the latter is the more important. Later work by Davies and Law (1987) showed that their values for the weight fraction of κ-casein were likely to be overestimated by about 0.02, but this correction hardly affects the calculation of $\bar{t}$. If the decrease in α_{s2}-casein in late lactation is also brought about in whole or in part by plasmin action (Andrews and Alichanidis 1983; Le Bars and Gripon 1989), then the retention of PCs as smaller phosphopeptides would help to raise $\bar{t}$ to the nearly constant average value seen in early and middle lactation milks. Accordingly, $\bar{t}$ was assumed to be constant with the bulk milk value of 1.42.

The analytical results of White and Davies (1958) for the milk salts were converted to mole per litre concentrations for use in the thermodynamic model. Subsequent work has shown that the citrate results were systematically underestimated and values were therefore corrected by the average systematic errors reported by White and Davies (1963).

Figure 4 reveals some of the interrelationships among the total, non-diffusible and ultrafiltrate concentrations in these data, illustrating some of the original correlations reported by White and Davies (1958). Figure 4a shows that there is a general tendency for ultrafiltrate $[P_i]$ to be negatively correlated with pH and for the pH of late lactation and mastitis milk to be generally higher than in early or middle lactation. In late lactation and during mastitic infection the mammary epithelium becomes more permeable to small ions and even proteins and an exchange of constituents can occur, one effect of which is to raise the milk pH (Shennan and Peaker 2000). In purely physicochemical terms, the effect of raised pH is to increase the non-diffusible multivalent ion concentrations at the expense of the diffusible ones. Nevertheless, Fig. 4a shows that superimposed on these physiological and physicochemical effects is a considerable natural variation in ultrafiltrate $[P_i]$ at any pH. Most notable among the effects on the salt partition is the effect of Cit. Because of the strong complex formed between the divalent cations and Cit^{3-}, positive correlations among $[Ca]_u$, $[Mg]_u$ and $[Cit]_u$ are commonly observed in milks (Fig. 4b). Figure 4c shows the close correlation of $[Ca]_c$ or $[Mg]_c$ with $[P_i]_c$, giving a regression line with a slope of 1.68 for the former.

Application of the thermodynamic model to the milks allows the ultrafiltrate composition to be predicted and compared with experiment. As can be seen in Fig. 5, the

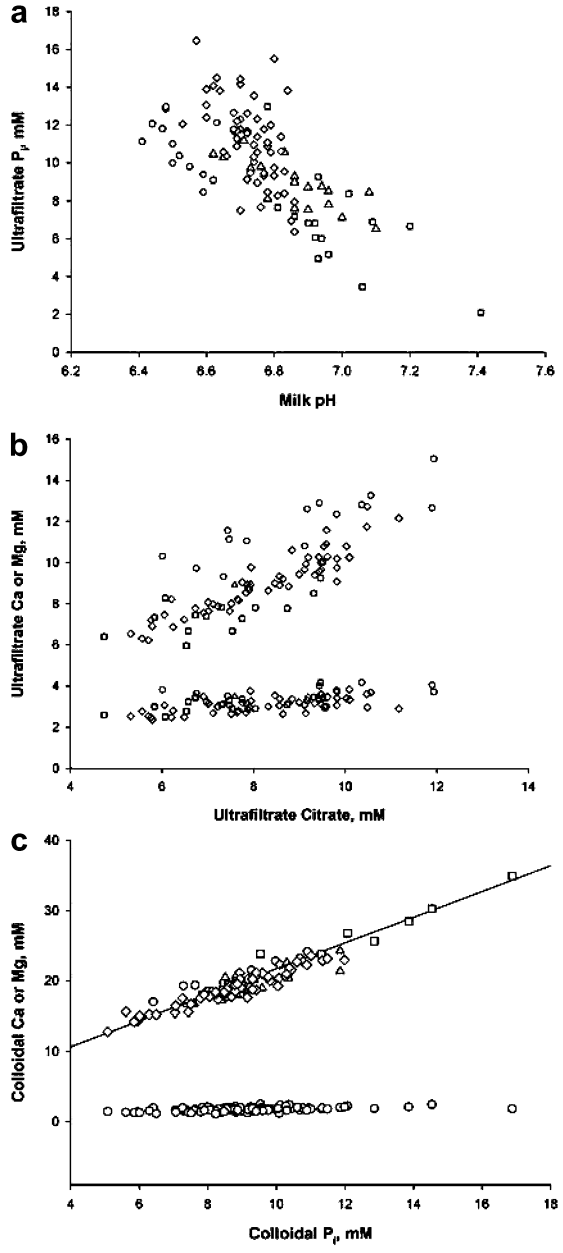


Fig. 4a–c Interrelationships of salt concentrations in the milks of individual Ayrshire cows reported by White and Davies (1958) but recalculated as molar concentrations (Table 6). Samples are from healthy cows in early (*circles*), middle (*diamonds*) or late (*squares*) lactation or from cows with mastitis (*triangles*). (**a**) Ultrafiltrate P_i versus milk pH. (**b**) Ultrafiltrate Ca or Mg versus ultrafiltrate Cit. (**c**) Non-diffusible Ca or Mg versus non-diffusible P_i

calculated and experimental results are very highly correlated, as demonstrated by the linear least-squares regression lines with slopes close to unity. The root mean square differences between calculation and theory were 0.448 ± 0.408, 0.340 ± 0.272, 1.451 ± 0.829 and 0.246 ± 0.167 mM for $[P_i]_u$, $[Cit]_u$, $[Ca]_u$ and $[Mg]_u$, respectively. Whereas it would be possible to match calculation with experiment more closely by adjusting one or more parameters of the model, this might disguise any shortcomings in representing real milks.

Calcification of the mammary gland and the stability of milk

The condition for stability of a milk (Eq. 24) requires that nanocluster-like calcium phosphate particles are able to form and therefore that there should be an excess of PCs over the concentration required to form the nanocluster-like MCP ($\alpha < 1$). It is also desirable for there to be a finite concentration of unreacted PCs to inhibit the phase separation of crystalline calcium phosphate phases such as DCPD. In computing the salt partition, the extent of reaction was allowed to exceed unity. The normalized histogram of α values for early and mid lactation milks (Fig. 6a) shows, however, that the great majority fall within the stable region ($\alpha < 1$). In contrast, in late lactation milks and in those classified as mastitic, the occurrence of unstable milks is three times higher. Within the healthy groups the trend is for α to increase with stage of lactation and pH and the mastitic group has the highest average pH. Thus, the prediction of the model is that uncontrolled precipitation of calcium phosphate becomes increasingly likely with advancing lactation and is likely to be at its worst during involution of the gland. The predictions correspond with clinical and pathological observations that the calcium phosphate milk stones, *corpora amylacea*, are seldom seen in healthy primiparous cows or women but their occurrence increases with age and parity (Niewold et al. 1999).

Optimal nutrition, or at least adequacy, has often provided the framework for discussing the biological function of milk constituents, but it has little to offer in explaining the primary or secondary structure of the caseins. Indeed, whey proteins have a higher content of essential amino acids than casein. Control of calcification, on the other hand, has much to recommend it since caseins are extremely effective, through their rheomorphic structure and PCs, in preventing calcification. The mammary gland is one of the most active transporters of Ca in mammalian biology and yet the occurrence of pathological calcification seldom threatens either lactation or feeding. This is no doubt due to the effectiveness of the caseins since, in all milks known to science, there is a balance between casein secretion and Ca content, as required by the stability criterion expressed through Eq. (24). Moreover, it is worth noting that in cows' milk, at least, there is a certain economy in the secretion of casein since the extent of reaction of the PCs is, on average, about 90%. Thus the milk composition is comfortably in the range that allows the formation of thermodynamically stable CPN-like particles.

Partition of caseins

There is no satisfactory data on the partition of caseins with which the theory can be compared. Holt et al. (1986) dissociated casein micelles slowly by a dialysis procedure to reduce the non-diffusible salt concentrations and

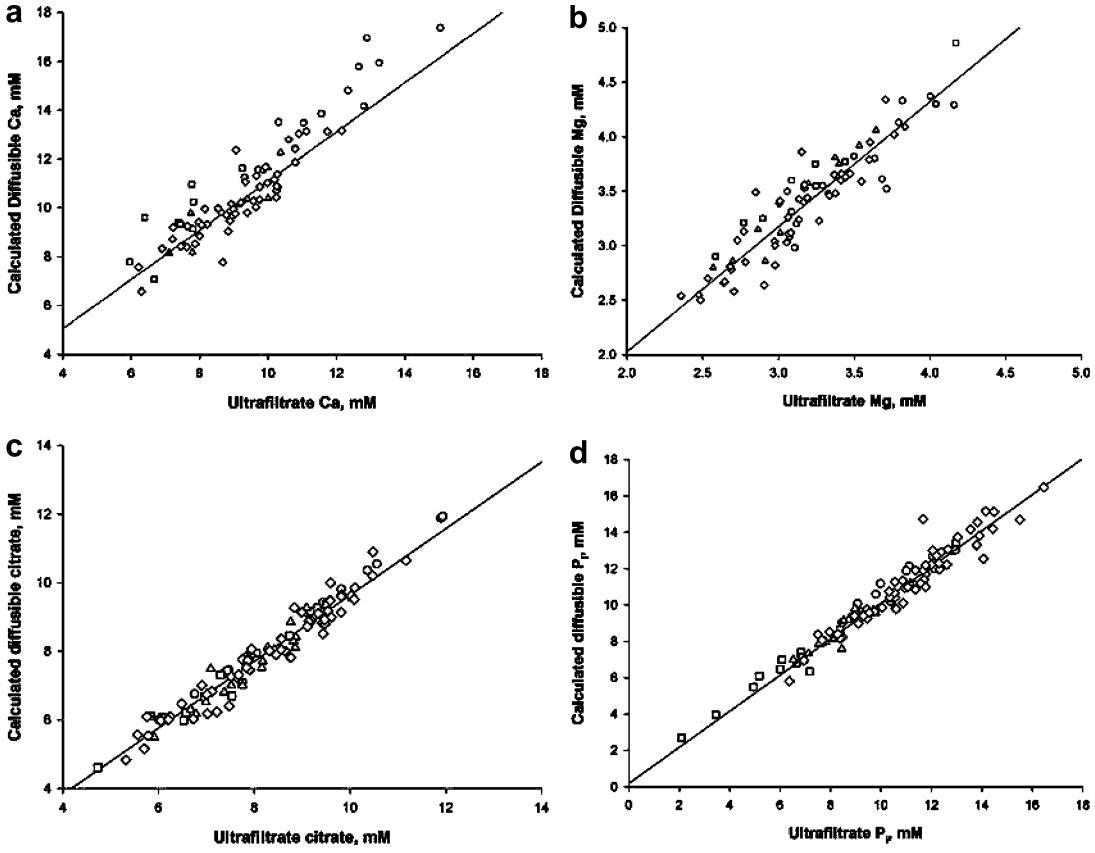
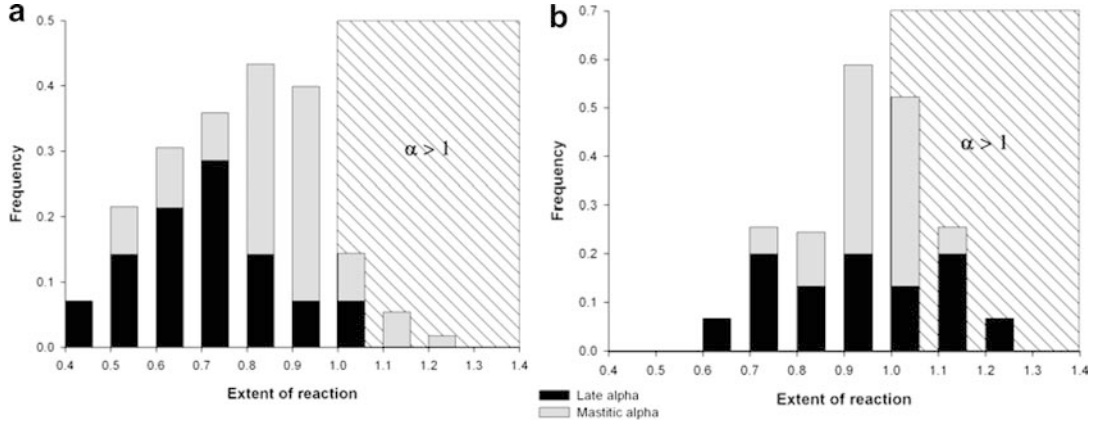


Fig. 5a–d Comparison of model calculations of diffusible concentrations with experimental ultrafiltrate values. Symbols are the same as those used in Fig. 4. (**a**) Ca, $y = -0.368 + 1.220x$; $r^2 = 0.787$. (**b**) Mg, $y = -0.655 + 1.314x$; $r^2 = 0.833$. (**c**) Cit, $y = -0.292 + 1.002x$; $r^2 = 0.955$. (**d**) P_i, $y = 0.557 + 0.964x$; $r^2 = 0.951$

partitioned the casein into a centrifugal pellet and supernatant serum. The ease of dissociation was β-, κ- $> \alpha_{s1}$- $> \alpha_{s2}$-casein, more-or-less in accord with the order shown in Fig. 2b. However, the dissociation was into particles, not monomeric proteins, and the size of the supernatant particles increased with the degree of dissociation (Holt 1998). Thus, the pelleting method did not measure a partitioning of casein comparable to the theoretical prediction. A similar experiment where MCP was reduced by acidification showed that the caseins do not dissociate from the micelle as readily as they do at normal milk pH because of the reduced micellar charge (Dalgleish and Law 1988, 1989).

More promising perhaps are experiments reporting the dissociation of almost pure β-casein from casein micelles on cooling to about 4 °C. Creamer et al. (1977) produced the first good evidence of the dynamics of exchange of β-casein in native casein micelles, observing an exchange of up to 26% of the total protein in 24 h at 4 °C with an exchange time in the order of several hours.

Fig. 6 Histogram of α values in (**a**) early (*light*) and middle (*dark*) lactation and (**b**) late lactation (*light*) and milks from cows with mastitis (*dark*). The *hatched zone* corresponds to the zone of instability where $\alpha \geq 1$. In each of the four groups, the number of occurrences in each α range was normalized by dividing by the total number of samples in that group



Likewise, when milk was cooled to 4 °C for increasing lengths of time, and the micelles pelleted by ultracentrifugation, an increasing proportion of the β-casein remained in the supernatant (Davies and Law 1983) up to a limit of about 40% of the total β-casein after 40 h. Re-equilibration was completely reversible at 20 °C in 18 h. Both experiments therefore suggest that an equilibrium partition between serum and micelles exists for the fraction of β-casein not bound to the calcium phosphate in the micelles. In contrast to the partition of β-casein, none of the other caseins showed an appreciable dependence on temperature or were accumulated in appreciable amounts in the serum. Assuming all PCs are equivalent, the experiments of Creamer et al. (1977) and Davies and Law (1983) give values for the extent of reaction of 0.74 and 0.60, respectively.

Validity of the equilibrium thermodynamic model

There are three key principles involved in applying the equilibrium model to the calculation of the milk partition, each of which may eventually require modification. These are that: (1) MCP is essentially the same as CPN apart from some unimportant differences relating to the distribution of PCs along the casein polypeptide chains; (2) the formation of the nanocluster core allows an equilibrium to be established between it and the continuous phase; and (3) the nanoclusters are of essentially constant composition. In addition to these key assumptions, there are some other aspects that are more likely to affect the precision than the principle of the partition calculation, such as the assumptions about the important equilibria among the small ions and the method of approximating the binding of Ca and Mg ions, in particular, to unreacted PCs.

In regard to the first principle, the experimental evidence is that the size, structure and, essentially also, the composition of MCP and CPN are indistinguishable. It may transpire that the nature of the polypeptide chain does play some part in determining the thermodynamic stability of nanoclusters or the reactivity of the PCs such that the centres cannot be considered equal and independent. If they are not all equivalent, then the principal effect will be seen in the partition of the caseins rather than the salts. Well designed experiments bearing on this point have not yet been performed.

The second principle is empirical in that an IAP has been observed in milk and nanocluster-forming solutions, with only inorganic constituents required for its expression. An IAP is normally taken as evidence of an equilibrium between two phases, but here it reflects the equilibrium with the nanocluster core, where the calcium phosphate is not in its standard state (Little and Holt 2004). If the activity of the core depends on the nature of the shell, then there will not be universal constants governing the partition of milk salts.

The third principle can be justified from experiments done on CPN solutions. Although these complexes appear to exist as a distribution of sizes, their average size (and hence, composition) is very little affected by wide variations in solution composition. Further experiments will be required to investigate how sensitive they are to the nature of the phosphopeptide.

In summary, the thermodynamic model based on the formation of nanoclusters provides a reasonable prediction of the partition of milk salts without introducing any arbitrary assumptions into the calculation. Nor has it required the parameters of the theory to be adjusted in order to obtain agreement with experiment. Figure 5 shows that the simple model provides a very nice correspondence of experiment with theory and further refinement may not be required until complete milk salt and casein compositional data are obtained on the same milks.

Thermodynamic stability of milk

It has long been recognized that milk salts, in particular divalent cations and the pH, are important factors in determining milk processing behaviour, particularly in influencing the stability of the protein in aggregation, gelation and precipitation reactions, and in the fouling of heat exchanger surfaces with mineralized deposits. Perhaps there is some benefit to be gained from restating the problem the other way round. This is that casein provides a vital way of converting an intrinsically unstable milk salt system into one that is thermodynamically stable and reversible under a wide range of conditions. It achieves this by forming CPN-like particles within the matrix of the casein micelle, using the casein PCs, rather than allowing a macroscopic phase of calcium phosphate to form. This effectiveness exists in cows' milk only over a limited pH range, 5.8–7.0. Below pH 5.8, the casein can hardly influence the behaviour of the salts, and phase separation of TCC, among other effects, can occur. Above about pH 7, the PCs have all reacted and further CPN structures can no longer form. An initial ACP phase can then nucleate and mature into highly insoluble and poorly crystalline basic calcium phosphates. In like manner, when Ca is added to milk, free casein PCs can only be effective if their concentration is high enough to form the nanoclusters. The fundamental rule of stability is encapsulated in Eq. (24). Thus milk becomes intrinsically unstable unless a balance is maintained between the casein and the Ca salt concentrations that leaves some free PCs.

Fouling of heat exchanger surfaces with a mineral-rich deposit during milk processing provides an obvious example where the control of phase separation by casein micelles fails. This may be because an initial deposit on the metal surface of denatured serum proteins occurs, which is effectively impermeable to the casein micelles but permeable to the small ions. As a result, a zone of instability develops close to the metal surface since there are no PCs to form nanoclusters. Prolonged running of the heat exchanger results in the formation of a calcium phosphate-rich deposit.

Our understanding of the milk salts has advanced to the point where precise, physicochemical models give good predictions of the partition of salts in milk and account quantitatively for the interrelationships that occur as a result of natural variations in composition. The most important point to note is that a balance is needed between casein and salts if the milk is to be stable and if the mammary gland is not to suffer pathological calcification as a result of lactation. Such a balance is possible through the remarkable properties of casein, once thought of as a random coil protein with only a nutritional function.

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