14
The Self-Assembly and Structure of Caseins in Solution

GORDON A. MORRIS*

Division of Food Sciences, School of BioSciences, University of Nottingham, Sutton Bonington, LE12 5RD, UK

Introduction

Milk proteins have been studied extensively in biochemistry for over 150 years. Milk is itself a complex mixture of water, lactose, fat, minerals and proteins. The protein composition of milk varies greatly from one species to another, concentrations range from 10–200 g l^{-1} (Jennes, 1970). Bovine milk has generated the most interest, although caprine, equine, ovine, and human milks have also been investigated (Carroll *et al.*, 1985; Sood *et al.*, 1985, 1992; Ono *et al.*, 1989; Sood and Slattery, 1997, 2000; Mora-Gutierrez *et al.*, 1998; Guerardel *et al.*, 1999). Bovine milk on average consists of 3–4% protein (Jennes, 1970; Dickinson and Stainsby, 1982). Historically, milk proteins have been split into two types, depending on their acid solubility:

i) acid insoluble caseins
ii) acid soluble whey proteins.

Both the caseins and whey proteins consist of multi-component mixtures.

Caseins are the major protein components of bovine milk and are found in concentrations of approximately (27 ± 2) g l^{-1} (varies from breed to breed, as well as from cow to cow, depending on production techniques and seasonal changes) and constitute about 78% of total milk protein (*Table 14.1*) (Jennes, 1970). For more information on milk composition and genetic variation, the reader is referred to McMeekin (1970), Jennes (1970, 1982), Swaisgood (1982, 1992), Ng-Kwai-Hang and Grosclaude (1992) and references therein.

*To whom correspondence may be addressed (g.morris@irl.cri.nz). Present address: Carbohydrate Chemistry Group, Industrial Research Limited, PO Box 31-310, Gracefield Rd., Lower Hutt, New Zealand.

Abbreviations: DP, degree of polymerization; S, Svedberg unit (10^{-13}sec); SAXS, small angle X-ray scattering; CMP, caseinomacropeptide; MHKS, Mark-Houwink-Kuhn-Sakurada coefficients.

Biotechnology and Genetic Engineering Reviews – Vol. 19, July 2002
0264–8725/02/19/357–376 $20.00 + $0.00 © Intercept Ltd, PO Box 716, Andover, Hampshire SP10 1YG, UK

Table 14.1. Some properties of the major components of bovine milk (adapted from Jennes, 1970 – with kind permission of Academic Press). Assumes total protein = 36 g l^{-1} and ~ 78% casein.

Protein	Approximate Concentration, g l^{-1}	Genetic Variants	Monomer M_w[a], g mol^{-1}	$s_{20,w}$[a], S
Caseins	28.0			
α_s-Casein	15.4	A, B, C, D	25 000	1.6
β-Casein	7.0	A^1, A^2, A^3, B, B_2, C, D	24 100	1.4
κ-Casein	4.2	A, B	20 000	1.57
γ-Casein	1.4	A, B	30 000	1.55
Whey proteins	8.0			
β-Lactoglobulin	4.8	A, B, C, D	18 300	1.8
α-Lactalbumin	1.1	A, B	14 200	1.75
Bovine serum albumin	0.5		69 000	4.0
Immunoglobulin G	0.9		160 000	7.0

[a]Values not necessarily corrected for non-ideality.

Caseins are defined as the group of phosphoproteins from milk which are precipitated at pH 4.6 and 20°C and were first separated in the 1950s (Hipp *et al.*, 1952; Von Hippel and Waugh, 1955; Waugh and Von Hippel, 1956; Long *et al.*, 1958; Hipp *et al.*, 1961). This group of molecules has been found using starch-gel electrophoresis to consist of more than 20 different species (see McKenzie, 1971 and references therein), all of which may be true components of the system (Thompson, 1971). Caseins have a tendency to self-associate or interact with one another in the form of 'casein micelles' and 'casein sub-micelles', a process considered entropically driven due to hydrophobic interactions (Fang and Dalgleish, 1996; Dickinson and Golding, 1997). The amphoteric nature of the molecules also allows for electrostatic interactions (Euston *et al.*, 1996).

This review will attempt to elucidate the solution properties (in terms of molecular weight, sedimentation coefficient, hydrodynamic radius, intrinsic viscosity, axial ratio and voluminosity/hydration) of the major caseins (α_{s1}-, α_{s2}-, β- and κ-) which underpin the functional properties of milk and technology of milk-based products. It will also consider how the individual properties of these casein types – particularly of the α- and β-caseins – can lead to a more informed picture of casein aggregation products, namely the casein micelle and the casein sub-micelle. As Thompson (1971) once put it: 'To understand the construction of the total unit, one must first concern oneself with the nature of those components (and the forces which lead to interaction) comprising the total unit'.

The principal casein fractions

The principal casein fractions, namely α_{s1}-, α_{s2}-, β- and κ-casein, constitute approximately 30–40%, 10–15%, 25–40% and 10–15% of bovine casein respectively (Jennes, 1970; Eigel *et al.*, 1984; Grappin and Ribadeau-Dumas, 1992; Rollema, 1992; Srinivasan *et al.*, 1996). The common compositional factor between all these is that they are all conjugated proteins (McKenzie and Murphy, 1970), most with phosphate group(s) esterified to serine residues (Richardson *et al.*, 1992; Swaisgood, 1992).

These phosphate groups play an important role in the structure and stability of the casein micelle. Calcium binding by the individual caseins is proportional to the phosphate content (Swaisgood, 1992).

The conformations of caseins have previously been likened to that of denatured globular proteins due to the unusually high number of proline residues (Farrell Jr., 1973; Kumosinski *et al.*, 1991a,b), namely 17, 10, 35 and 20 for α_{s1}-, α_{s2}-, β- and κ-casein respectively, which prevent the formation of ordered secondary structures (Guo *et al.*, 1995). However, a recent review into the chemistry of caseins (Swaisgood, 1992), together with the work of Andrews *et al.* (1979) and Kumosinski *et al.* (1991a,b), have suggested a more detailed structure with at least some secondary structure to a level perhaps as high as 70–80% for κ-casein (Swaisgood, 1992). This evidence for secondary and tertiary structure had been obtained from Raman spectroscopy and X-ray diffraction studies of crystalline molecules: however, it must be noted that casein molecules do not form crystals.

This lack of secondary and tertiary structure has lead to various attempts to estimate the structure of caseins in solution, and in this review particular attention will be paid to solution properties of bovine caseins but, where applicable, the properties of other mammalian caseins will also be discussed.

α_{S1}-Casein

This has a monomer molecular weight 23 000 g mol^{-1}, with 199 amino acid residues, 17 of which are proline. The amino acid sequence for α_{s1}-casein, from SWISS-PROT entry P02662 has been found to be:

RPKHPIKHQG LPQEVLNENL LRFFVAPFPE VFGKEKVNEL SKDIGSESTE
DQAMEDIKQM EAESISSSEE IVPNSVEQKH IQKEDVPSER YLGYLEQLLR
LKKYKVPQLE IVPNSAEERL HSMKEGIHAQ QKEPMIGVNQ ELAYFYPELF
RQFYQLDAYP SGAWYYVPLG TQYTDAPSFS DIPNPIGSEN SEKTTMPLW

It consists of two hydrophobic regions, containing all the proline residues, separated from one another by a polar region, containing seven of the eight phosphate groups. It can be precipitated at very low levels of calcium and undergoes ionic strength dependent self-association; the mechanism is believed to be a step-wise addition (Rollema, 1992), i.e.

$$(\alpha_{s1}\text{-casein})_n + \alpha_{s1}\text{-casein} \rightarrow (\alpha_{s1}\text{-casein})_{n+1}$$

The structure of the α_{s1}-casein monomer is presented in *Figure 14.1*. As with other caseins, it undergoes self-association, which for α_{s1}-casein – and also α_{s2}-casein – is in a step-wise manner, i.e. monomers to dimers to trimers, etc. The degree of association depends on the pH and ionic strength of the solution (Swaisgood, 1992) and the association product was estimated at pH 6.7, ionic strength 0.2 M and 35°C to have a molecular weight (molar mass) of 3 400 000 g mol^{-1} (Thurn *et al.*, 1987b). The hydrodynamic properties are therefore quite different to those of the other caseins. Swaisgood (1992) attempted to estimate the hydration and the asymmetry (axial ratio of the equivalent prolate ellipsoid) from the measured intrinsic viscosity, [η], of 10 ml g^{-1}. By assuming a so-called 'typical' globular protein hydration value of 0.3–0.5, he

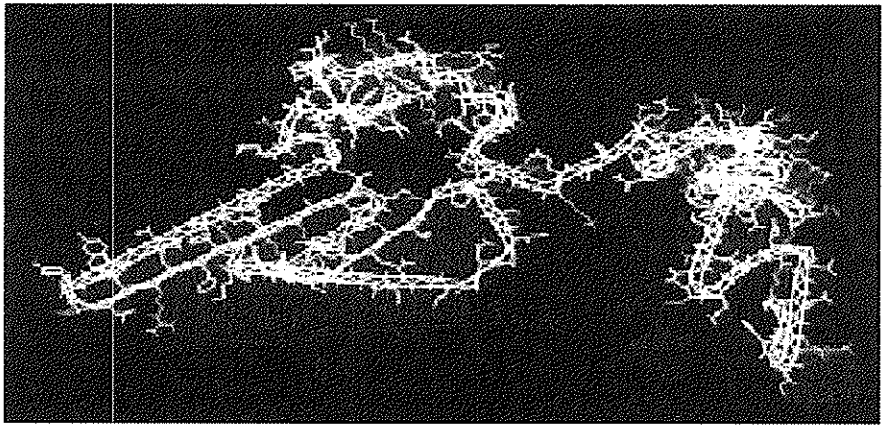

Figure 14.1. Three-dimensional molecular model for an α_{s1}-casein monomer (Kuminoski *et al.*, 1991b). Reproduced with kind permission of the American Dairy Science Association.

calculated an axial ratio, a/b of 7. On the other hand, if he had assumed a spherical monomer (a/b = 1), this results in a hydration of approximately 3–3.5. An alternative estimate comes from sedimentation analysis in the analytical ultracentrifuge and measurement of the translational frictional ratio, f/f_0 (Tanford, 1961; Harding, 1997, Morris, 2001):

$$\frac{f}{f_0} = \frac{M_w(1-\bar{\nu}\,\rho_{20,w})}{(N_A 6\pi\eta_{20,w} s^0_{20,w})\left(\frac{4\pi N_A}{3\bar{\nu}M_w}\right)^{-1/3}} \qquad (14.1a)$$

Here M_w is the weight average molecular weight (g mol^{-1}); $\bar{\nu}$ is the partial specific volume (ml g^{-1}); $\rho_{20,w}$ is the density of water at 20°C (0.99823 g ml^{-1}); N_A is Avogadro's number (6.023 × 10^{23} mol^{-1}); $\eta_{20,w}$ is the viscosity of water at 20°C (0.01002 Poise) and $s^0_{20,w}$ (sec) is the infinite dilution sedimentation coefficient corrected to standard solvent conditions of the density and viscosity of water at 20°C. The measured value for f/f_0 of 1.16 from a weight average molecular weight of 23 000 g mol^{-1} and a sedimentation coefficient of 2.4 S is consistent with a more globular protein of average hydration – in truth, it seems likely that the 'real' structure may be somewhere in between the viscosity and sedimentation results, and perhaps not so different to that suggested by Kuminoski *et al.* (1991b).

α_{s2}-Casein

This has a monomer molecular weight 25 000 g mol^{-1}, with 207 amino acid residues including 10 prolines. The amino acid sequence for α_{s2}-casein has been obtained from the SWISS-PROT entry P02663 and is:

KNTMEHVSSS EESIISQETY KQEKNMAINP SKENLCSTFC KEVVRNANEE
EYSIGSSSEE SAEVATEEVK ITVDDKHYQK ALNEINQFYQ KFPQYLQYLY

QGPIVLNPWD QVKRNAVPIT PTLNREQLST SEENSKKTVD MESTEVFTKK TKLTEEEKNR LNFLKKISQR YQKFALPQYL KTVYQHQKAM KPWIQPKTKV IPYVRYL

Negatively charged residues are found clustered near the N-terminus and positively charged residues near the C-terminus: this, therefore, is the most hydrophilic of the caseins (Swaisgood, 1992). Unlike α_{s1}-casein and β-casein, it may contain a disulphide bond (α_{s1}-casein and β-casein haven no cysteine residues). It is also very calcium sensitive. It undergoes a similar ionic strength dependent self-association to α_{s1}-casein (Rollema, 1992), although it has been reported to associate to a lesser extent, due to greater electrostatic repulsion and reduced hydrophobicity (Swaisgood, 1992):

$$(\alpha_{s2}\text{-casein})_n + \alpha_{s2}\text{-casein} \rightarrow (\alpha_{s2}\text{-casein})_{n+1}$$

There are limited hydrodynamic data available for the monomer. Its intrinsic viscosity is 11.4 ml g^{-1} which Swaisgood (1992) has claimed to be consistent with a hydrodynamic radius of 3.6 nm, a hydration of 3.9 and a shape factor (ν) of ~ 9 (Harding *et al.*, 1982), all of which are consistent with his calculations for α_{s1}-casein, although in the absence of any supporting data from measurement of sedimentation coefficients in the analytical ultracentrifuge.

β-Casein

β-Casein is the second most abundant casein, at approximately 25–40% (Jennes, 1970; Eigel *et al.*, 1984; Grappin and Ribadeau-Dumas, 1992; Rollema, 1992; Srinivasan *et al.*, 1996) of the total casein content in bovine milk. It has a monomer molecular weight 24 000 g mol^{-1}, with 209 amino acid residues, including 35 prolines. The amino acid sequence for β-casein has been obtained from the SWISS-PROT entry P02666 and is:

RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE AMAPKHKEMP FPKYPVEPFT ESQSLTLTDV ENLHLPLPLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY PQRDMPIQAF LLYQEPVLGP VRGPFPIIV

It has a highly charged, N-terminal region and a hydrophobic C-terminal region. It is, thus, a very amphiphilic protein, and acts like a detergent molecule: it is no surprise, therefore, that it is widely used as an emulsifier. It is less sensitive to calcium precipitation than the α_s-caseins. Its tendency is to self-associate into a large discrete polymer (Rollema, 1992):

$$n\ (\beta\text{-casein}) \rightarrow (\beta\text{-casein})_n$$

β-Casein self-association is concentration, temperature and ionic strength dependent (Sullivan *et al.*, 1955; Payens and Van Markwijk, 1963; Payens *et al.*, 1969; Andrews *et al.*, 1979; Arima *et al.*, 1979; Buchheim and Schmitt, 1979; Evans *et al.*, 1979; Morris, 2001). Most studies on such phenomena have been undertaken at lower

temperatures, e.g. 8.5 and 13.5°C (Payens and Van Markwijk, 1963) in order to facilitate the determination of the monomer molecular weight and sedimentation coefficient (24 000 g mol^{-1} and 1.6 S respectively). Although significant aggregation takes place at 20°C (Hoagland, 1966; Payens *et al.*, 1969; Andrews *et al.*, 1979; Arima *et al.*, 1979; Buchheim and Schmitt, 1979; Evans *et al.*, 1979; Morris, 2001), there is still a large amount of monomer present, especially at low concentration (Sullivan *et al.*, 1955; Payens and Van Markwijk, 1963; Hoagland, 1966; Thompson, 1971; Arima *et al.*, 1979; Buchheim and Schmitt, 1979; Morris, 2001).

A typical sedimentation velocity experiment shows two distinct peaks (*Figure 14.2*): one for the monomer and the other for a larger polymer (Sullivan *et al.*, 1955; Payens and Van Markwijk, 1963; Hoagland, 1966; Thompson, 1971; Arima *et al.*, 1979; Morris, 2001), this is consistent with the bimodal distribution found by Buchheim and Schmitt (1979) from electron microscopy. The 'slow' component has been found to have a sedimentation coefficient of 1.6 S, which is typical of a β-casein monomer; the larger species (~ 12.0 S) is a significantly larger polymer. The sedimentation coefficient for the 'fast' moving species has been reported to have a strong concentration dependency (Payens and Van Markwijk, 1963; Payens *et al.*, 1969; Thompson, 1971) at the concentrations required (~ 2–3 g l^{-1}) for the Schlieren optical system on the ultracentrifuge. The relative amount of the polymer (%) decreases with decreasing concentration (Payens and Van Markwijk, 1963; Morris, 2001); this is further quantified by a decrease in the weight average sedimentation coefficient (Morris, 2001).

Owing to the problems of temperature-dependent and ionic strength-dependent self-association, the molecular weights and degrees of polymerization calculated for bovine β-casein, not surprisingly, have been found to depend greatly on experimental conditions (Payens *et al.*, 1969; Andrews *et al.*, 1979; Arima *et al.*, 1979; Buchheim and Schmitt, 1979; Takase *et al.*, 1980; Thurn *et al.*, 1987a; Morris, 2001). Such behaviour was reviewed by Rollema (1992): the degree of polymerization (DP) has been shown to vary from about 10 to up to 60, although at room temperature a DP between 40 to 50 has been found to be most prevalent (Swaisgood, 1992).

β-CASEIN MONOMER

Having calculated the molecular weight and sedimentation coefficient, it is, therefore, possible to estimate the translational frictional ratio from *Equation 14.1a* or an equivalent equation from molecular weight and the translational diffusion coefficient (Tanford, 1961):

$$\frac{f}{f_0} = \frac{k_B T}{6\pi\eta_{20,w}} \left(\frac{4\pi N_A}{3\bar{v} M_w}\right)^{1/3} \frac{1}{D^0_{20,w}} \qquad (14.1b)$$

where k_B is the Boltzmann constant (1.381×10^{-16} erg K^{-1} mol^{-1}) and $D^0_{20,w}$ is the infinite dilution diffusion coefficient corrected to standard conditions of 20°C and water (cm^2 sec^{-1}).

The frictional ratio for the monomer has been calculated to be approximately 1.8 (Sullivan *et al.*, 1955; Payens and Van Markwijk, 1963; Thompson, 1971; Andrews *et al.*, 1979; Sood and Slattery, 1997; Morris, 2001) and corresponds to a hydro-

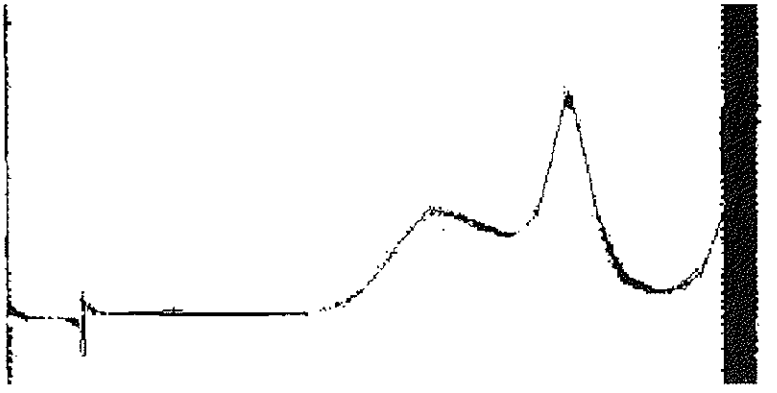

Figure 14.2. Typical sedimenting boundary for β-casein at 8.5°C in barbiturate buffer pH 7.5, I = 0.2 M (reprinted from *Biochimica et Biophysica Acta* **71**, Payens and Van Markwijk, Some Features of the Association of β-Casein, 517–530, copyright (1963) with kind permission of Elsevier Science).

dynamic radius, r_H of about 3.6 nm (Schmitt and Payens, 1972; Andrews *et al.*, 1979; Swaisgood, 1992). An intrinsic viscosity of 23 ml g^{-1} (Payens and Van Markwijk, 1963) has been determined. The sedimentation coefficient, diffusion coefficient, frictional ratio, intrinsic viscosity and hydrodynamic radius found in this way are all typical of an extended and/or unusually hydrated molecule (see for example Tanford, 1961; Harding, 1997). Results from small angle X-ray scattering (SAXS) (Andrews *et al.*, 1979) point to a radius of gyration, R_g of 4.6 nm, which is smaller than the value of approximately 5.1 nm calculated from amino acid sequence (*Equation 14.2a*), sedimentation coefficient (*Equation 14.2b*) and intrinsic viscosity (*Equation 14.2c*) for a random coil molecule of 24 100 g mol^{-1} (209 amino acids), which would indicate at least some ordered secondary structure, the ratio of R_g/r_H would be expected to be 1.5 for random coil (Burchard, 1985).

$$6r_G^2 = (70 \pm 15)n \tag{14.2a}$$

$$s^0_{20,w} = \frac{M_w(1 - \bar{v}\rho_{20,w})}{4\pi N_A \eta_{20,w} r_G} \tag{14.2b}$$

$$[\eta] = \frac{2.23\pi N_A r_G^3}{M_w} \tag{14.2c}$$

where n is the number of amino acids in the protein molecule. This may be consistent with a Wales–Van Holde ratio, $R = k_s/[\eta]$ (see Wales and Van Holde, 1954; Rowe, 1977) of 1.35 (Andrews *et al.*, 1979; Swaisgood, 1992), which points to an axial ratio, a/b of 2.6 (calculated using the ELLIPS1 program, Harding and Cölfen, 1995); this is somewhat different from the value of 12 suggested by Swaisgood (1992) but on the assumption that β-casein hydration is similar to that of globular proteins, 0.3–0.5. It should be noted that the Wales–Van Holde ratio used to obtain the value of 2.6 is *independent* of hydration. Knowledge of the Wales–Van Holde ratio can then allow us to estimate the values for the Perrin function, P (see Harding, 1997), the Einstein

viscosity increment, ν (see Einstein, 1911 and Harding, 1997) and the hydration, δ from *Equations 14.3a, 14.3b* and *14.3c*.

$$P = \left(\frac{f}{f_0}\right)\left[\frac{\bar{v}}{v_s}\right]^{1/3} \tag{14.3a}$$

$$[\eta] = \nu v_s \tag{14.3b}$$

$$v_s = \bar{v} + \frac{\delta}{\rho_{20,w}} \tag{14.3c}$$

where v_s is the swollen volume (ml g^{-1}); $[\eta]$ is the intrinsic viscosity (ml g^{-1}). This allows an estimate of hydration of approximately 7 from viscosity (ν = 3.4) and ~ 3 from sedimentation experiments (P = 1.1). Although the values of δ from sedimentation and viscometry are quite different, they do appear to be in line with the previous estimates of 2 – 8 from viscometry (Swaisgood, 1992; Boulet *et al.*, 1998) and from sedimentation (Sood *et al.*, 1992 – human β-casein).

Although hydrodynamic techniques suggest that β-casein monomers are moderately extended and hydrated molecules, when imaged by electron microscopy measurements, the β-casein monomers appear as spheres of approximately 4 nm in radius (Andrews *et al.*, 1979). Swaisgood (1982) attempted to explain this unusual phenomenon by suggesting that the β-casein molecule 'contains regions of stable structure, although large regions have marginal stability and exhibit a large degree of segmental motion' and are therefore prone to exhibit random, coil-like behaviour in solution (Swaisgood, 1982; Boulet *et al.*, 1998). The fact that electron microscopical images do not correspond to true aqueous solution conditions may be, at least in part, responsible for the difference. This aside, it appears that, in solution, the β-casein monomer is a moderately extended and extremely hydrated molecule.

β-CASEIN POLYMER

Due to the large inconsistencies in molecular weight determinations for the β-casein polymer (300 000–1 400 000 g mol^{-1} at 20–25°C) (Rollema, 1992), it is very difficult to get a definitive answer on conformation – the general consensus appears to be that the β-casein polymer is spherical (Andrews *et al.*, 1979; Buchheim and Schmitt, 1979; Evans *et al.*, 1979; Thurn *et al.*, 1987a; Sood *et al.*, 1992), although Payens and Van Markwijk (1969) and Morris (2001) favour a more extended model.

κ-Casein

κ-Casein has a monomer molecular weight 19 000 g mol^{-1}, with 169 amino acid residues, including 20 prolines. The amino acid sequence for κ-casein with the rennet cleavage site in bold (Jollès *et al.*, 1962) has been obtained from the SWISS-PROT entry P02668 and is:

QEQNQEQPIR CEKDERFFSD KIAKYIPIQY VLSRYPSYGL NYYQQKPVAL
INNQFLPYPY YAKPAAVRSP AQILQWQVLS NTVPAKSCQA QPTTMARHPH

PHLSFMAIPP KKNQDKTEIP TINTIASGEP TSTPTTEAVE STVATLEDSP EVIESPPEIN TVQVTSTAV

It has been found to be very resistant to calcium precipitation and stabilizes other caseins. Rennet cleavage at the **Phe105–Met106** bond has been found to eliminate this stabilizing ability (MacKinlay and Wake, 1965, 1971; MacKinlay *et al.*, 1966; McKenzie, 1971; Kumosinski *et al.*, 1991a; Anema, 1997; Farrell Jr. *et al.*, 1998; Lehner *et al.*, 1999), leaving the hydrophobic portion, para-kappa-casein, and a hydrophilic portion called caseinomacropeptide (CMP). Concentration-dependent aggregation forms discrete polymers.

$$n\ (\kappa\text{-casein}) \rightarrow (\kappa\text{-casein})_n$$

The proposed structure for a κ-casein is presented in *Figure 14.3*.

κ-Casein is the least abundant of the four major caseins (α_{s1}, α_{s2}, β and κ) and is present at about 10–15% in bovine milk (Jennes, 1970; Eigel *et al.*, 1984; Grappin and Ribadeau-Dumas, 1992; Rollema, 1992; Srinivasan *et al.*, 1996). Although κ-casein (and other caseins) have not been crystallized, molecular models based on amino acid sequence have been proposed (Kumosinski *et al.*, 1991a,b) (*Figure 14.3*). Such structures have been described as the 'horse and rider', the most recent model providing good correlation with κ-casein functional properties (Kumosinski *et al.*, 1991a). The most interesting property is that κ-casein appears calcium insensitive (Waugh and Van Hippel, 1956) and is therefore suspected to play a key role in micellular stabilization (Waugh and Van Hippel, 1956; Sullivan *et al.*, 1959; Swaisgood and Brunner, 1962, 1963; Thompson and Pepper, 1962; Pepper and Thompson, 1963; Zittle and Walter, 1963; MacKinlay and Wake, 1964, 1965; Swaisgood *et al.*, 1964; Rose, 1965; MacKinlay *et al.*, 1966; Payens, 1966; Rose and Colvin, 1966a,b; Thompson *et al.*, 1967; Waugh *et al.*, 1970; Farrell Jr., 1973; Dewan *et al.*, 1974; Carroll and Farrell Jr., 1983; Kumosinski *et al.*, 1991a; Anema, 1997; Farrell Jr. *et al.*, 1998; Groves *et al.*, 1998; Lehner *et al.*, 1999). Evidence that κ-casein is involved in micelle stabilization is due to the fact that it is insensitive to calcium precipitation in the concentrations normally found in milk (Zittle and Walter, 1963). κ-Casein is also the milk component most sensitive to the action of the enzyme chymosin (referred to as rennin in many publications) (MacKinlay and Wake, 1965, 1971; MacKinlay *et al.*, 1966; McKenzie, 1971; Kumosinski *et al.*, 1991a; Farrell Jr. *et al.*, 1998; Lehner *et al.*, 1999). Chymosin cleaves the κ-casein molecule at the Phe-Met bond (105–106) and releases the 'hairy tail' – casein macropeptide (CMP) – and leaving the insoluble para-κ-casein (Kumosinski *et al.*, 1991a; Farrell Jr. *et al.*, 1998; Lehner *et al.*, 1999). This destroys the micelle stabilizing ability and results in micelle coagulation. Heat-induced coagulation of micelles is due to the interaction of κ-casein with β-lactoglobulin at high temperatures (Zittle *et al.*, 1962). Various authors have also suggested that the degree of phosphorylation, disulphide bonding and glycosylation of κ-casein also play minor roles in micellular stabilization (Thompson and Pepper, 1962; Pepper and Thompson, 1963; Zittle and Walter, 1963; MacKinlay and Wake, 1964, 1965; Hoagland, 1966; Farrell Jr., 1973; Kumosinski *et al.*, 1991a; Farrell Jr. *et al.*, 1998).

The mechanism of stabilization is still somewhat vague. In the classical model

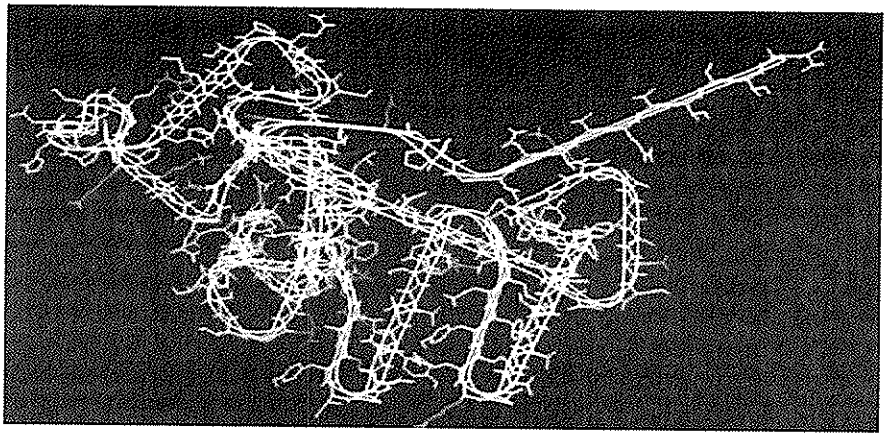

Figure 14.3. Three-dimensional molecular model for a κ-casein monomer (Kuminoski *et al.*, 1991a). Reproduced with kind permission of the American Dairy Science Association.

suggested by Waugh (Waugh *et al.*, 1970; Waugh, 1971), the κ-casein is proposed to concentrate on the outside of the micelle, the core consisting of a hydrophobic α_s-β complex. Carroll and Farrell Jr. (1983) have subsequently brought this theory into doubt; they suggest instead that there may be two different types of micelles:

i) κ-casein at the periphery
ii) κ-casein uniformly distributed throughout micelle.

They further claim that micelles with uniformly distributed κ-casein are smaller than those with κ-casein concentrated on the micellular surface. This may be related to the findings of Sullivan *et al.* (1959), Rose (1965), Rose and Colvin (1966b), McGann and Pyne (1970) and Devold *et al.* (2000), who all claim that increased κ-casein contents decrease micelle size.

κ-Casein is known to self-associate, and this behaviour has been assayed in many solvents, including phosphate (various concentrations/pHs), 5 M guanidine hydrochloride, 7 M urea, acetic acid (33, 66%), NaCl (Swaisgood and Brunner, 1962, 1963; Thompson and Pepper, 1962; Pepper and Thompson, 1963; Zittle and Walter, 1963; MacKinlay and Wake, 1964; Swaisgood *et al.*, 1964; Farrell Jr. *et al.*, 1998; Morris, 2001). However, most authors have been concerned with calculating the molecular weight of monomeric species. The consensus molecular weight and sedimentation coefficient are 23 000 g mol^{-1} and 1.4 S respectively: the molecular weight is in agreement with that calculated from the amino acid composition (Kumosinski *et al.*, 1991a). Under conditions similar to those of milk (approximately pH 7 and ionic strength 0.1 M), a single sedimentation coefficient of ~ 13.5 S has been measured (Pedersen, 1936; Swaisgood and Brunner, 1962, 1963; Thompson and Pepper, 1962; Pepper and Thompson, 1963; Zittle and Walter, 1963; MacKinlay and Wake, 1964; Swaisgood *et al.*, 1964; Morris, 2001). It should be noted that for the paper by Pedersen (1936), the current naming convention for caseins was not in use and, therefore, the species characterized had not been designated κ-casein as such.

Molecular weight determinations have been somewhat less frequent. Swaisgood *et al.* (1964) suggest a molecular weight of 650 000 g mol^{-1} for the 13.5 S species; Thurn *et al.* (1987a) calculate a molecular weight of 2 500 000 g mol^{-1}; Farrell Jr. *et al.* (1998) suggest 650 000–1 200 000 g mol^{-1} at 25°C and 895 000–2 000 000 g mol^{-1} at 37°C, and Morris (2001) determined a polymer molecular weight of approximately 750 000 g mol^{-1}. In the determinations of Farrell Jr. *et al.* (1998), the differences in calculated molecular weights are dependent on the analysis program used. This does, however, show that the temperature and concentration dependency of aggregation can cause significant problems and inconsistencies in sedimentation equilibrium analyses. It can therefore be inferred that the κ-casein molecular weight is anywhere upward of 600 000 g mol^{-1}, although a value of 600 000–900 000 g mol^{-1} at 20°C would not be an unreasonable estimate.

Assuming, therefore, a molecular weight of 750 000 g mol^{-1} and sedimentation coefficient of 13.8 S, it is possible to estimate a translational frictional ratio of 2.1 (Tanford, 1961) (*Equation 14.1a*). One can also estimate the diffusion coefficient (1.7×10^{-7} cm^2 sec^{-1}) by rearrangement of the *Equation 14.1b*; results would suggest that κ-casein is a moderately extended and/or hydrated molecule. A Wales–Van Holde ratio of 1.6 is consistent with a spherical molecule (k_s = 15.9 ml g^{-1} – Morris, 2001 and $[\eta]$ = 9.5 ml g^{-1} – Swaisgood *et al.*, 1964) and would therefore suggest that the non-unity value of the translational frictional ratio is due to hydration alone.

Casein micelles

A large proportion of casein in milk is in the form of casein micelles, which can be thought of as a complex of calcium caseinate–calcium phosphate (citrate) (Dickinson and Stainsby, 1982). The casein micelle is usually thought of as a hydrated sphere, with the 'hydration' – δ (i.e. the amount of solvent associated with the protein either chemically or physically entrained expressed as mass of water per unit mass of protein) estimated on the basis of dynamic light scattering (Dewan *et al.*, 1973) to be (3.7 ± 0.5) and from sedimentation velocity and capillary viscometry (Morris *et al.*, 2000; Morris, 2001) to be (3.4 ± 0.5). The colloidal calcium phosphate is essential for micellular stability. Removal of Ca^{2+} ions results in a smaller complex – the casein 'sub-micelle'. κ-Casein is usually found on the exterior of the casein sub-micelle and therefore important to sub-micellular and micellular stability (Waugh and Von Hippel, 1956; Sullivan *et al.*, 1959; Swaisgood and Brunner, 1962, 1963; Thompson and Pepper, 1962; Pepper and Thompson, 1963; Zittle and Walter, 1963; MacKinlay and Wake, 1964, 1965; Swaisgood *et al.*, 1964; Rose, 1965; MacKinlay *et al.*, 1966; Payens, 1966; Rose and Colvin, 1966a,b; Thompson *et al.*, 1967; Waugh *et al.*, 1970; Farrell Jr., 1973; Dewan *et al.*, 1974; Carroll and Farrell Jr., 1983; Kumosinski *et al.*, 1991a; Farrell Jr. *et al.*, 1998; Groves *et al.*, 1998; Lehner *et al.*, 1999). The κ-casein is thought to coat the hydrophobic core of the sub-micelle (*Figure 14.4*) and is important in casein/polysaccharide interactions due to it having a positively charged region available for electrostatic bonding (Snoeren *et al.*, 1976; Dalgleish and Morris, 1988; Drohan *et al.*, 1997; Augustin *et al.*, 1999; Bourriot *et al.*, 1999, 2000; Langendorff *et al.*, 1999, 2000a,b; see also Rollema, 1992). In recent years, the casein sub-micelle model has been modified and a more open model with a hairy κ-casein outer layer has been suggested (De Kruif and Zhulina, 1996; Holt and Horne, 1996;

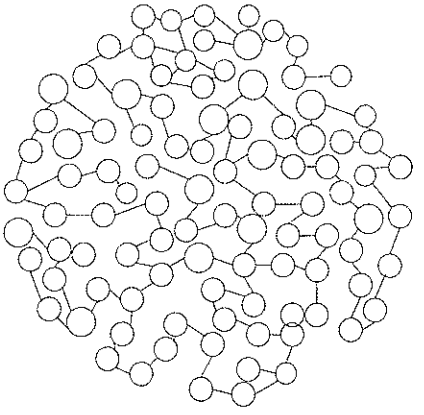

Figure 14.4. Sub-micelle model for the casein micelle (calcium phosphate bridges link individual sub-micelles).

De Kruif, 1999; see also Rollema, 1992). It has recently been reported (Jonkman *et al.*, 1999a,b) that the behaviour and structure of casein micelles does not change significantly upon processing, i.e. ice cream manufacture.

The size distribution of casein micelles is very broad (20–250 nm in diameter) (McKinnon *et al.*, 1999; Morris, 2001), and this is at least partially due to the availability of κ-casein (Sullivan *et al.*, 1955; Rose and Colvin, 1966b; McGann and Pyne, 1970; Dickinson and Stainsby, 1982).

The sedimentation profile of casein micelles (*Figure 14.5*) reveals a broad peak, i.e. large size distribution, and shows a large concentration dependency (Morris *et al.*, 2000; Morris, 2001). The mode average sedimentation coefficient, $s^0_{T,b} = (845 \pm 2)$ S and $k_s = (16.9 \pm 0.1)$ ml g^{-1}, this is characteristic of a large, extremely hydrated spherical molecule (Morris *et al.*, 2000; Morris, 2001) (see *Table 14.2*). The Wales–Van Holde ratio, $R = k_s[\eta]$ (see e.g. Creeth and Knight, 1965; Rowe, 1977, 1992; Harding, 1997): this combination of the Gralén parameter, k_s (Gralén, 1944) and the intrinsic viscosity $[\eta]$ can give an approximate concentration independent indication of macromolecular shape, which is independent of an assumed value of the hydration, δ, as we have already noted above.

In the same study, Morris (2001) calculated an intrinsic viscosity of 10.4 ml g^{-1} and the Wales–Van Holde ratio was therefore calculated to be 1.6. Since the theoretical value for a sphere is 1.6 (see e.g. Creeth and Knight, 1965; Rowe, 1977, 1992; Harding, 1997), it is reasonable to say that casein micelles are ~ spherical. Having confirmed the spherical shape, it is possible to calculate the hydration (*Equations 14.3b* and *14.3c*) using the Einstein viscosity increment, ν, where ν is 2.5 for a sphere (Einstein, 1911; Harding, 1997). This yields a value of 4.2 ml g^{-1} for the swollen specific volume; this results in a hydration, δ of (3.4 ± 0.5) grams of solvent/grams of protein, which is in good agreement with the value of (3.7 ± 0.5) (Dewan *et al.*, 1973) from dynamic light scattering.

It is also possible to estimate the molecular weight from the sedimentation coefficient, $s^0_{20,w}$, the Gralén parameter, k_s, and the swollen volume, v_s (Rowe, 1977) (*Equation 14.4*).

$$M_w = N_A \left[\frac{6\pi_{20,w}\eta_{20,w}s^0_{20,w}}{\left(1-\bar{v}\rho_{20,w}\right)} \right]^{3/2} \left[\left(\frac{3\bar{v}}{4\pi}\right)\left(\frac{k_s}{2\bar{v}} - \frac{v_s}{\bar{v}}\right) \right]^{1/2} \quad (14.4)$$

From *Equation 14.4* a molecular weight, M_w of 2.8×10^8 g mol^{-1}, can be calculated from the sedimentation coefficient of 845 S (Morris *et al.*, 2000; Morris, 2001). The value for the molecular weight calculated from this equation is in good agreement with the value of 2.0×10^8 g mol^{-1} (Schorsch *et al.*, 1999a,b) measured by turbidity, and would seem consistent with a spherical species of diameter of approximately 150 nm (McKinnon *et al.*, 1999). Further simple mathematical manipulation of the data can yield the corresponding diffusion coefficient, $D^0_{20,w}$ and hydrodynamic radius, r_H, via rearrangements of the Svedberg and Stokes–Einstein (see e.g. Harding, 1999) relationships (*Equations 14.5a* and *14.5b* respectively).

$$\frac{s^0_{20,w}}{D^0_{20,w}} = \frac{M_w(1-\bar{v}\rho_{20,w})}{RT} \quad (14.5a)$$

$$r_H = \frac{k_B T}{6\pi\eta_{20,w}D^0_{20,w}} \quad (14.5b)$$

The calculated $D^0_{20,w} = 2.8 \times 10^{-8}$ cm^2 sec^{-1} and $r_H = 78$ nm are also in good agreement with the previously published values (Lin *et al.*, 1971; Dewan *et al.*, 1974; Schorsch *et al.*, 1999b). Morris (2001) further confirmed this estimation of $D^0_{20,w}$ (and hence molecular weight) by dynamic light scattering. It is also possible to calculate the translational frictional ratio, f/f_0 (Tanford, 1961) from either *Equations 14.1a* or *14.1b*, which is reported to be 1.8.

Morris (2001) continued to presume that casein micelles are spherical (i.e. the MHKS exponent from sedimentation, b = 0.666) and formulated the following MHKS relationship – $s_{20,w} = 2.0 \times 10^{-3} M_w^{0.666}$, which is in agreement with the equation calculated by Dewan *et al.* (1974) – $s_{20,w} = 1.8 \times 10^{-3} M_w^{0.665}$.

Although apparently more complex, it seems as if the hydrodynamic properties of the casein micelle may well be better understood than those of the individual components (especially α_{s1}- and α_{s2}-casein).

Discussion

Although widely studied in the last 150 years, there are still many questions to be answered with regard to the solution structure of the caseins. It would seem that, with respect to casein micelles, the structure is quite clear, the properties of the individual caseins are somewhat mysterious; this is especially true for the much under-studied α_s-caseins. On the other hand, the wealth of data available for β-casein only seems to have confused the issue further. It may be that the instability theory of Swaisgood (1992) may require greater attention. In the case of κ-casein, discrepancies in polymer molecular weight are at least partially due to data analysis (Farrell Jr. *et al.*, 1998).

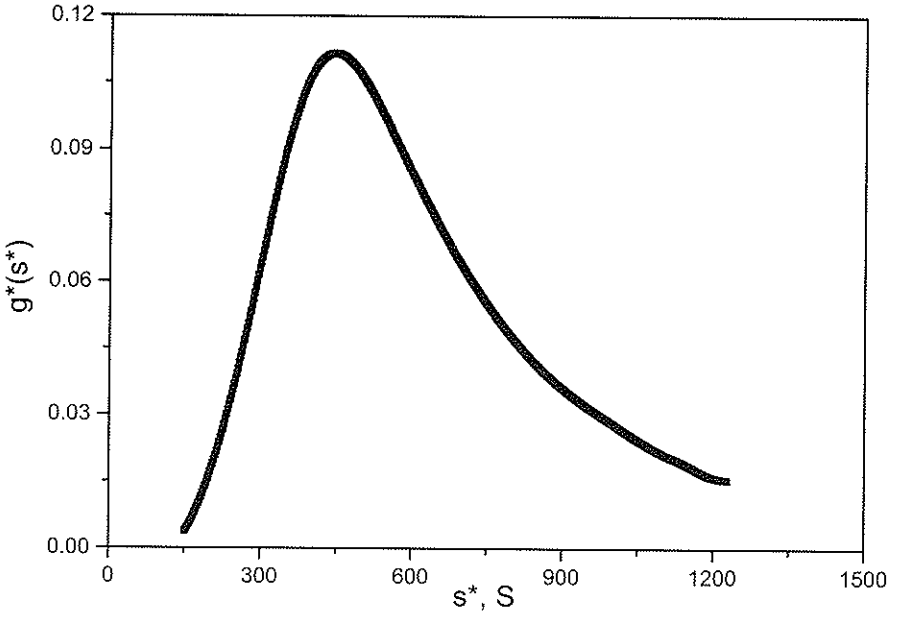

Figure 14.5. g*(s*) profile for casein micelles at 0.028 g l^{-1}, i.e. the approximate concentration in bovine milk (adapted from Morris *et al.*, 2000 and Morris, 2001, with kind permission of the American Chemical Society). Where the * is indicative of an *apparent* sedimentation coefficient distribution, i.e. not corrected for diffusion, and an *apparent* sedimentation coefficient, i.e. not corrected for non-ideality and not necessarily to standard solvent conditions (Stafford, 1992a,b; Laue and Stafford, 1999). Conditions: 20°C; I ~ 0.1 M; rotor speed 12 600 rpm (Beckman Optima XLI with Rayleigh Interference optics).

Table 14.2. Hydrodynamic parameters for casein micelles (adapted from Morris *et al.*, 2000 – with kind permission of the American Chemical Society).

Parameter	Value
ν, ml g^{-1}	0.733
$s^0_{20,w}$, S	845
k_s, ml g^{-1}	16.9
$D^0_{20,w}$ cm^2 sec^{-1}	2.8×10^{-8}
[η], ml g^{-1}	10.4
$k_s/[\eta]$	1.6
P	1.0
ν	2.5
a/b	1.0
δ	3.4
ν_s, ml g^{-1}	4.2
M_w, g^{-1} mol	2.8×10^8
r_H, nm	78
f/f_0	1.8

ν, calculated from average amino acid composition (Perkins, 1986).

It would appear that, contrary to the views of Thompson (1971), it is not always necessary to understand the individual components to understand the whole. This, then, also begs the question: 'Do the structures of the individual caseins alone actually have any bearing on the structure of the casein micelle (or sub-micelle)?' In the context of the current literature, this author would be inclined to answer in the negative.

References

ANDREWS, A.L., ATKINSON, D., EVANS, M.T.A., FINER, E.G., GREEN, J.P., PHILLIPS, M.C. AND ROBERTSON, R.N. (1979). The conformation of bovine β-casein A. I. Molecular aspects of thermal aggregation. *Biopolymers* **18**, 1105–1121.

ANEMA, S.C. (1997). The effect of chymosin on κ-casein coated polystyrene latex particles and bovine casein micelles. *International Dairy Journal* **7**, 553–558.

ARIMA, S., NIKI, R. AND TAKASE, K. (1979). Structure of β-casein. *Journal of Dairy Research* **46**, 281–282.

AUGUSTIN, M.A., PUVANENTHIRAN, A. AND MCKINNON, I.R. (1999). The effect of κ-carrageenan conformation on its interaction with casein micelles. *International Dairy Journal* **9**, 413–414.

BOULET, M., BRITTEN, M. AND LAMARCHE, F. (1998). Voluminosity of some food proteins in aqueous dispersions at various pH and ionic strengths. *Food Hydrocolloids* **12**, 433–441.

BOURRIOT, S., GARNIER, C. AND DOUBLIER, J.-L. (1999). Micellular casein – κ-carrageenan mixtures I. Phase separation and ultrastructure. *Carbohydrate Polymers* **40**, 145–157.

BOURRIOT, S., GARNIER, C. AND DOUBLIER, J.-L. (2000). A description of micellular casein/κ-carrageenan mixed systems by means of calorimetry and rheology. In: *Gums and stabilisers for the food industry 10*. Eds. G.O. Phillips and P.A. Williams, pp 203–210. Cambridge: Royal Society of Chemistry.

BUCHHEIM, W. AND SCHMITT, D.G. (1979). On the monomers and polymers of β-casein. *Journal of Dairy Research* **46**, 277–280.

BURCHARD, W. (1985). New aspects of polymer characterization by dynamic light scattering. *Chimia* **39**, 10–18.

CARROLL, R.J. AND FARRELL, H.M. JR. (1983). Immunological approach to location of κ-casein in the casein micelles by electron microscopy. *Journal of Dairy Science* **66**, 679–686.

CARROLL, R.J., BASCH, J.J., PHILLIPS, J.G. AND FARRELL, H.M. JR. (1985). Ultrastructural and biochemical investigations of mature human milk. *Food Microstructure* **4**, 323–331.

CREETH, J.M. AND KNIGHT, C.G. (1965). On the estimation of the shape of macromolecules from sedimentation and viscosity measurements. *Biochimica et Biophysica Acta* **102**, 549–558.

DALGLEISH, D.G. AND MORRIS, E.R. (1988). Interactions between carrageenans and casein micelles: electrostatic and hydrodynamic properties of the particles. *Food Hydrocolloids* **2**, 311–320.

DE KRUIF, C.G. (1999). Casein micelles interactions. *International Dairy Journal* **9**, 183–188.

DE KRUIF, C.G. AND ZHULINA, E.B. (1996). κ-Casein as a polyelectrolyte brush on the surface of casein micelles. *Colloids and Surfaces A: Physiochemical and Engineering Aspects* **17**, 151–159.

DEVOLD, T.G., BROVOLD, M.J., LANGSRUD, T. AND VEGARUD, G.E. (2000). Size of native and heated casein micelles, content of protein and minerals in milk from *Norwegian Red Cattle* – effect of milk polymorphism and different feeding regimes. *International Dairy Journal* **10**, 313–323.

DEWAN, R.K., BLOOMFIELD, V.A., CHUDGAR, A. AND MORR, C.V. (1973). Viscosity and voluminosity of bovine milk casein micelles. *Journal of Dairy Science* **56**, 699–705.

DEWAN, R.K., CHUDGAR, A., MEAD, R., BLOOMFIELD, V.A. AND MORR, C.V. (1974). Molecular weight and size distribution of bovine milk casein micelles. *Biochimica et Biophysica Acta* **342**, 313–321.

DICKINSON, E. AND GOLDING, M. (1997). Depletion flocculation of emulsion containing unadsorbed sodium caseinate. *Food Hydrocolloids* **11**, 13–18.

DICKINSON, E. AND STAINSBY, G. (1982). *Colloids in food*. London: Applied Science Publishers.

DROHAN, D.D., TZIBOULA, A., MCNULTY, D. AND HORNE, D.S. (1997). Milk protein – carrageenan interactions. *Food Hydrocolloids* **11**, 101–107.

EIGEL, W.N., BUTLER, J.E., ERNSTROM, C.A., FARRELL, H.M. JR., HARWALKAR, V.R., JENNES, R. AND WHITNEY, R.M. (1984). Nomenclature of proteins of cow's milk. Fifth revision. *Journal of Dairy Science* **75**, 3432–3451.

EINSTEIN, A. (1911). Correction to my paper: 'A new determination of molecular dimensions' Berichtigung zu meiner arbeit: 'Eine neue bestimmung der molekuldimensionen'. *Annalen der Physik* **34**, 591–593.

EUSTON, S.E., SINGH, H., MUNRO, P.A. AND DALGLEISH, D.G. (1996). Oil in water emulsions stabilized by sodium caseinate or whey protein isolate as influenced by glycerol monostearate. *Journal of Food Science* **61**, 916–920.

EVANS, M.T.A., PHILLIPS, M.C. AND JONES, M.N. (1979). The conformation of bovine β-casein A. II. Thermodynamics of thermal association and the effects of changes in polar and apolar interactions on micellization. *Biopolymers* **18**, 1123–1140.

FANG, Y. AND DALGLEISH, D.G. (1996). Competitive adsorption between dioleoylphosphatidycholine and sodium caseinate on oil–water interfaces. *Journal of Agriculture and Food Chemistry* **44**, 59–64.

FARRELL, H.M. JR (1973). Models for casein micelle formation. *Journal of Dairy Science* **56**, 1195–1206.

FARRELL, H.M. JR., WICKHAM, E.D. AND GROVES, M.L. (1998). Environmental influences on purified κ-casein: disulfide interactions. *Journal of Dairy Science* **81**, 2974–2984.

GRALÉN, N. (1944). Sedimentation and diffusion measurements on cellulose and cellulose derivatives. PhD Dissertation, University of Uppsala, Sweden.

GRAPPIN, R. AND RIBADEAU-DUMAS, B. (1992). Analytical methods for milk proteins. In: *Advanced dairy chemistry. Vol 1: Proteins*. Ed. P.F. Fox, pp 1–62. New York: Elsevier Science.

GROVES, M.L., WICKHAM, E.D. AND FARRELL, H.M. JR. (1998). Environmental effects on disulfide bonding patterns of bovine κ-casein. *Protein Chemistry* **17**, 73–84. (Abstract)

GUERARDEL, Y., MORELLE, W., PLANCKE, Y., LEMOINE, J. AND STRECKER, G. (1999). Structural analysis of three oligosaccharides isolated from human milk. *Carbohydrate Research* **320**, 230–238.

GUO, M.R., FOX, P.F., FLYNN, A. AND KINDSTEDT, P.S. (1995). Susceptibility of β-lactoglobulin and sodium caseinate to proteolysis by pepsin and trypsin. *Journal of Dairy Science* **78**, 2336–2344.

HARDING, S.E. (1997). The intrinsic viscosity of biological macromolecules. Progress in measurement, interpretation and application to structure in dilute solution. *Progress in Biophysics and Molecular Biology* **68**, 207–262.

HARDING, S.E. (1999). Protein hydrodynamics. In: *Protein structure: a comprehensive treatise, Vol. 2*. Ed. G. Allen, pp 271–305. Greenwich, Connecticut: JAI Press.

HARDING, S.E. AND CÖLFEN, H. (1995). Inversion formulae for ellipsoid of revolution macromolecular shape functions. *Analytical Biochemistry* **228**, 131–142.

HARDING, S.E., DAMPIER, M.D. AND ROWE, A.J. (1982). The viscosity increment for ellipsoids of revolution – some observations on the Simha formula. *Biophysical Chemistry* **15**, 205–208.

HIPP, N.J., GROVES, M.L., CUSTER, J.H. AND MCMEEKIN, T.L. (1952). Separation of α-, β- and γ-casein. *Journal of Dairy Science* **35**, 272–280.

HIPP, N.J., GROVES, M.L. AND MCMEEKIN, T.L. (1961). Separation of the components of α-casein II. The preparation of α_3-casein. *Archives in Biochemistry and Biophysics* **93**, 245–250.

HOAGLAND, P.D. (1966). Acylated β-caseins: electrostatic interactions and aggregation. *Journal of Dairy Science* **49**, 783–787.

HOLT, C. AND HORNE, D.S. (1996). The hairy casein micelle: evolution of the concept and its implications for dairy technology. *Netherlands Milk and Dairy Journal* **50**, 85–111.

JENNES, R. (1970). Protein composition of milk. In: *Milk proteins: chemistry and molecular biology. Vol I.* Ed. H.A. McKenzie, pp 17–44. New York: Academic Press.

JENNES, R. (1982). Inter-species comparison of milk proteins. In: *Developments in dairy chemistry.* Vol 1. Ed. P.F. Fox, pp 87–114. London: Applied Science Publishers.

KUMOSINSKI, T.F., BROWN, E.M. AND FARRELL, H.M. JR. (1991a). Three dimensional modeling of bovine caseins: κ-casein. *Journal of Dairy Science* **74**, 2879–2887.

KUMOSINSKI, T.F., BROWN, E.M. AND FARRELL, H.M. JR. (1991b). Three dimensional modeling of bovine caseins: α_{s1}-casein. *Journal of Dairy Science* **74**, 2889–2895.

JOLLÈS, P., ALAIS, C. AND JOLLÈS, J. (1962). Amino acid composition of κ-casein and terminal amino acids of κ- and para-κ-casein. *Archives of Biochemistry and Biophysics* **98**, 56–57.

JONKMAN, M.J., WALSTRA, P., VAN BOEKEL, M.A.J.S. AND CEBULA, D.J. (1999a). Behaviour of casein micelles at conditions similar to those in frozen yoghurt. *International Dairy Journal* **9**, 385–386.

JONKMAN, M.J., WALSTRA, P., VAN BOEKEL, M.A.J.S. AND CEBULA, D.J. (1999b). Behaviour of casein micelles at conditions similar to those in ice cream. *International Dairy Journal* **9**, 401–405.

LANGENDORFF, V., CUVELIER, G., LAUNAY, B., MICHON, C., PARKER, A. AND DE KRUIF, C.G. (1999). Casein micelle/iota carrageenan interactions in milk: influence of temperature. *Food Hydrocolloids* **13**, 211–218.

LANGENDORFF, V., CUVELIER, G., MICHON, C., LAUNAY, B., PARKER, A. AND DE KRUIF, C.G. (2000a). Effects of carrageenan type on the behaviour of carrageenan/milk mixtures. *Food Hydrocolloids* **14**, 273–280.

LANGENDORFF, V., CUVELIER, G., MICHON, C., LAUNAY, B., PARKER, A. AND DE KRUIF, C.G. (2000b). Stability and gelation of carrageenan/skim milk mixtures: influence of temperature and carrageenan type. POLYMERIX 2000 – Biopolymers for the Food and Cosmetic Industry (Conference Proceeding), pp 147–155. Rennes, France: CBB Development.

LAUE, T.M. AND STAFFORD, W.F. III (1999). Modern applications of analytical ultracentrifugation. *Annual Reviews in Biophysics and Biomolecular Structure* **28**, 75–100.

LEHNER, D., WORNING, P., FRITZ, G., OGENDAL, L., BAUER, R. AND GLATTER, O. (1999). Characterisation of enzymatically induced aggregation of casein micelles in natural concentration by *in situ* static light scattering and ultra low shear viscometry. *Journal of Colloid and Interface Science* **213**, 445–456.

LIN, S.H.C., DEWAN, R.K., BLOOMFIELD, V.A. AND MORR, C.V. (1971). Inelastic light scattering study of the size distribution of bovine milk casein micelles. *Biochemistry* **10**, 4788–4793.

LONG, J., VAN WINKLE, Q. AND GOULD, L.A. (1958). Isolation and identification of λ-casein. *Journal of Dairy Science* **41**, 317–318.

MACKINLAY, A.G. AND WAKE, R.G. (1964). The heterogeneity of κ-casein. *Biochimica et Biophysica Acta* **93**, 378–386.

MACKINLAY, A.G. AND WAKE, R.G. (1965). Fractionation of S-carboxymethy-κ-casein and characterization of the components. *Biochimica et Biophysica Acta* **104**, 167–180.

MACKINLAY, A.G. AND WAKE, R.G. (1971). κ-Casein and it's attack by rennin (chymosin). In: *Milk proteins: chemistry and molecular biology. Vol II.* Ed. H.A. McKenzie, pp 175–216. New York: Academic Press.

MACKINLAY, A.G., HILL, R.J. AND WAKE, R.G. (1966). The action of rennin on κ-casein. The heterogeneity and origin of the insoluble products. *Biochimica et Biophysica Acta* **115**, 103–112.

MCGANN, T.C.A. AND PYNE, G.T. (1970). The colloidal phosphate of milk III. Nature of its association with casein. *Journal of Dairy Research* **27**, 403–417.

MCKENZIE, H.A. (1971). Whole casein: isolation, properties and zone electrophoresis. In: *Milk proteins: chemistry and molecular biology. Vol II.* Ed. H.A. McKenzie, pp 87–116. New York: Academic Press.

MCKENZIE, H.A. AND MURPHY, W.H. (1970). Methods for zone electrophoresis of milk protein. In: *Milk proteins: chemistry and molecular biology. Vol I.* Ed. H.A. McKenzie, pp 154–161. New York: Academic Press.

MCKINNON, I.R., BECKETT, R., UDABAGE, P. AND AUGUSTIN, M.A. (1999). The use of field

flow fractionation in the characterization of casein micelles. *International Dairy Journal* **9**, 391–392.

MCMEEKIN, T.L. (1970). Milk proteins in retrospect: In: *Milk proteins: chemistry and molecular biology. Vol II.* Ed. H.A. McKenzie, pp 3–16. New York: Academic Press.

MORA-GUTIERREZ, A., KUMOSINSKI, T.F. AND FARRELL, H.M. JR. (1998). κ-Carrageenan interaction with bovine and caprine caseins as shown by sedimentation and nuclear magnetic resonance spectroscopic techniques. *Journal of Agriculture and Food Chemistry* **46**, 4987–4996.

MORRIS, G.A. (2001). Hydrodynamic investigation of polysaccharides and their interactions with casein. PhD Dissertation, University of Nottingham, UK.

MORRIS, G.A., FOSTER, T.J. AND HARDING, S.E. (2000). The effect of degree of esterification on the hydrodynamic properties of citrus pectin. *Food Hydrocolloids* **14**, 227–235.

NG-KWAI-HANG, K.F. AND GROSCLAUDE, F. (1992). Genetic polymorphism of milk proteins. In: *Advanced dairy chemistry. Vol 1: Proteins.* Ed. P.F. Fox, pp 405–455. New York: Elsevier Science.

ONO, T., KOHNO, H., ODAGIRI, S. AND TAKAGI, T. (1989). Subunit components of casein micelles from bovine, ovine, caprine and equine milks. *Journal of Dairy Research* **56**, 61–68.

PAYENS, T.A.J. (1966). Association of caseins and their possible relation to the structure of the casein micelle. *Journal of Dairy Science* **49**, 1317–1324.

PAYENS, T.A.J. AND VAN MARKWIJK, B.W. (1963). Some features of the association of β-casein. *Biochimica et Biophysica Acta* **71**, 517–530.

PAYENS, T.A.J., BRINKHUIS, J.A. AND VAN MARKWIJK, B.W. (1969). Self-association in non-ideal systems. Combined light scattering and sedimentation measurements in β-casein solutions. *Biochimica et Biophysica Acta* **175**, 434–437.

PEDERSEN, K.O. (1936). Ultracentrifugal and electrophoretic studies on milk proteins. I. Introduction and preliminary results with fractions from skim milk. *Biochemical Journal* **30**, 948–960.

PEPPER, L. AND THOMPSON, M.P. (1963). Dephosphorylation of α_s- and κ-caseins and its effect on micelle stability in the κ-α_s-casein system. *Journal of Dairy Science* **46**, 764–767.

PERKINS, S.J. (1986). Protein volumes and hydration effects – the calculation of partial specific volumes, neutron-scattering matchpoints and 280 nm absorption-coefficients for proteins and glycoproteins from amino acid sequences. *European Journal of Biochemistry* **157**, 169–180.

RICHARDSON, T., OH, S., JIMÉNEZ-FLORES, R., KUMINOSKI, T.F., BROWN, E.M. AND FARRELL, H.M. JR. (1992). Molecular modeling and genetic engineering of milk proteins. In: *Advanced dairy chemistry. Vol 1: Proteins.* Ed. P.F. Fox, pp 545–578. New York: Elsevier Science.

ROLLEMA, H.S. (1992). Casein association and micelle formation. In: *Advanced dairy chemistry. Vol 1: Proteins.* Ed. P.F. Fox, pp 111–140. New York: Elsevier Science.

ROSE, D. (1965). Protein stability problems. *Journal of Dairy Science* **48**, 139–146.

ROSE, D. AND COLVIN, J.R. (1966a). Appearance and size of micelles from bovine milk. *Journal of Dairy Science* **49**, 1091–1097.

ROSE, D. AND COLVIN, J.R. (1966b). Internal structure of casein micelles from bovine milk. *Journal of Dairy Science* **49**, 351–355.

ROWE, A.J. (1977). Concentration-dependence of transport processes – general description applicable to sedimentation, translational diffusion, and viscosity coefficients of macromolecular solutes. *Biopolymers* **16**, 2595–2611.

ROWE, A.J. (1992). The concentration dependence of sedimentation. In: *Analytical ultracentrifugation in biochemistry and polymer science.* Eds. S.E. Harding, A.J. Rowe and J.C. Horton, pp 394–407. Cambridge: Royal Society of Chemistry.

SCHMITT, D.G. AND PAYENS, T.A.J. (1972). The evaluation of positive and negative contributions to the second virial coefficient of some milk proteins. *Journal of Colloid and Interface Science* **39**, 655–662.

SCHORSCH, C., CLARK, A.H., JONES, M.G. AND NORTON, I.T. (1999a). Behaviour of milk protein/polysaccharide systems in high sucrose. *Colloids and Surfaces B: Biointerfaces* **12**, 317–329.

SCHORSCH, C., JONES, M.G. AND NORTON, I.T. (1999b). Thermodynamic incompatibility and microstructure of milk protein/locust bean gum/sucrose systems. *Food Hydrocolloids* **13**, 89–99.

SNOEREN, T.H.M., BOTH, P. AND SCHMITT, D.G. (1976). An electron-microscopy study of carrageenan and its interaction with κ-casein. *Netherlands Milk and Dairy Journal* **30**, 132–141.

SOOD, S.M. AND SLATTERY, C.W. (1997). Monomer characterization and studies of self-association of the major β-casein of human milk. *Journal of Dairy Science* **80**, 1554–1560.

SOOD, S.M. AND SLATTERY, C.W. (2000). Association of the quadruply phosphorylated β-casein from human milk with the nonphosphoylated form. *Journal of Dairy Science* **83**, 2766–2780.

SOOD, S.M., CHANG, P. AND SLATTERY, C.W. (1985). Interactions in human casein systems: self-association of fully phosphorylated human β-casein. *Archives of Biochemistry and Biophysics* **242**, 355–364.

SOOD, S.M., CHANG, P. AND SLATTERY, C.W. (1992). Interaction properties of doubly phosphorylated β-casein, a major component of the human milk caseins. *Journal of Dairy Science* **75**, 2937–2945.

SRINIVASAN, M., SINGH, H. AND MUNRO, P.A. (1996). Sodium caseinate stabilized emulsions: factors affecting coverage and composition of surface proteins. *Journal of Agriculture and Food Chemistry* **44**, 3807–3811.

STAFFORD, W.F. III (1992a). Boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile. *Analytical Biochemistry* **203**, 295–301.

STAFFORD, W.F. III (1992b). Methods for obtaining sedimentation coefficient distributions. In: *Analytical ultracentrifugation in biochemistry and polymer science*. Eds. S.E. Harding, A.J. Rowe and J.C. Horton, pp 359–393. Cambridge: Royal Society of Chemistry.

SULLIVAN, R.A., FITZPATRICK, M.M., STANTON, E.K., ANNINO, R., KISSEL, G. AND PALERMITI, F. (1955). The influence of temperature and electrolytes upon apparent size and shape of α- and β-casein. *Archives of Biochemistry and Biophysics* **55**, 455–468.

SULLIVAN, R.A., FITZPATRICK, M.M. AND STANTON, E.K. (1959). Distribution of kappa-casein in skim milk. *Nature* **183**, 616–617.

SWAISGOOD, H.E. (1982). Chemistry of milk proteins. In: *Developments in dairy chemistry. Vol 1*. Ed. P.F. Fox, pp 1–59. London: Applied Science Publishers.

SWAISGOOD, H.E. (1992). Chemistry of the caseins. In: *Advanced dairy chemistry. Vol 1: Proteins*. Ed. P.F. Fox, pp 63–110. New York: Elsevier Science.

SWAISGOOD, H.E. AND BRUNNER, J.R. (1962). Characterisation of κ-casein obtained by fractionation with trichloroacetic acid in a concentrated urea solution. *Journal of Dairy Science* **45**, 1–11.

SWAISGOOD, H.E. AND BRUNNER, J.R. (1963). Characterisation of kappa-casein in the presence of various dissociating agents. *Biochemical and Biophysical Research Communications* **12**, 148–151.

SWAISGOOD, H.E., BRUNNER, J.R. AND LILLEVIK, H.A. (1964). Physical parameters of κ-casein from cow's milk. *Biochemistry* **3**, 1616–1623.

TAKASE, K., NIKI, R. AND ARIMA, S. (1980). A sedimentation equilibrium study of the temperature dependent association of bovine b-casein. *Biochimica et Biophysica Acta* **622**, 1–8.

TANFORD, C. (1961). *Physical chemistry of macromolecules*, Chapter 6. New York: John Wiley and Sons.

THOMPSON, M.P. (1971). α_s and β-caseins. In: *Milk proteins: chemistry and molecular biology. Vol II*. Ed. H.A. McKenzie, pp 117–174. New York: Academic Press.

THOMPSON, M.P. AND PEPPER, L. (1962). Effect of neurminidase on k-casein. *Journal of Dairy Science* **45**, 794–796.

THOMPSON, M.P., KALAN, E.B. AND GREENBERG, R. (1967). Properties of caseins modified by treatment with carboxypeptidase A. *Journal of Dairy Science* **50**, 767–769.

THURN, A., BURCHARD, W. AND NIKI, R. (1987a). Structure of casein micelles I. Small angle neutron scattering and light scattering from β- and κ-casein. *Colloid and Polymer Science* **265**, 653–666.

THURN, A., BURCHARD, W. AND NIKI, R. (1987b). Structure of casein micelles II. α_{s1}-casein. *Colloid and Polymer Science* **265**, 897–902.

VON HIPPEL, P.H. AND WAUGH, D.F. (1955). Casein monomers and polymers. *Journal of the American Chemical Society* **77**, 4311–4319.

WALES, M. AND VAN HOLDE, K.E. (1954). The concentration dependence of the sedimentation constants of flexible macromolecules. *Journal of Polymer Science* **14**, 81–86.

WAUGH, D.F. (1971). Formation and structure of casein micelles. In: *Milk proteins: chemistry and molecular biology. Vol II.* Ed. H.A. McKenzie, pp 3–86. New York: Academic Press.

WAUGH, D.F. AND VON HIPPEL, P.H. (1956). κ-Casein and the stabilization of casein micelles. *Journal of the American Chemical Society* **78**, 4576–4582.

WAUGH, D.F., CREAMER, L.K., SLATTERY, C.W. AND DRESDNER, G.W. (1970). Core polymers of casein micelles. *Biochemistry* **9**, 787–795.

ZITTLE, C.A. AND WALTER, M. (1963). Stabilization of β-casein by κ-casein against precipitation by calcium chloride. *Journal of Dairy Science* **46**, 1189–1191.

ZITTLE, C.A., THOMPSON, M.P., CUSTER, J.H. AND CERBULIS, J. (1962). κ-Casein–β-lactoglobulin interactions in solution when heated. *Journal of Dairy Science* **45**, 807–810.