Structural Studies on Casein Micelles of Human Milk: Dissociation of β-Casein of Different Phosphorylation Levels Induced by Cooling and Ethylenediaminetetraacetate

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ABSTRACT

Information on the structure of human casein micelles has been obtained from dissociation of β-casein (CN). Two approaches were used: cooling at 4°C and addition of EDTA. An initial loss of about 80% of the protein optical density occurred upon cooling to 4°C. Dissociation was time dependent, and at ≥24 h about 10% remained. However, mean size and voluminosity of micelles increased, as indicated by laser light scattering and viscosity measurements. This process was reversible, and 95% of the protein reentered the micelles upon incubation for 3 h at 37°C. Upon cooling, amounts of nonphosphorylated β-CN increased, and singly phosphorylated β-CN levels were almost constant relative to the total β-CN in micelles. Upon addition of EDTA (0 to 5 m*M*), the forms with three to five phosphates were the major dissociating constituents; EDTA that was added by dialysis produced similar results but at lower concentrations. These data suggest that, in the absence of significant amounts of α_{s1}-CN, nonphosphorylated and singly phosphorylated human β-CN may form a framework, as proposed for α_{s1}-CN for bovine milk, along with the colloidal calcium phosphate for the development of the final micelle structure by addition of the more highly phosphorylated forms. The results also indicate that human casein micelles have a less rigid structure than those of other species.
(**Key words**: human milk, casein micelles, micelle dissociation, micelle structure)

Abbreviation key: **P** = single protein phosphorylated (used with number to indicate degree of phosphorylation), **PMSF** = phenylmethylsulfonyl fluoride.

INTRODUCTION

Each mammalian species provides for the unique nutritional needs of the newborn by producing milk with a specific composition that reflects the requirements of the newborn. With this in mind, the manufacturers of infant formulas have attempted to match the nutritional composition of human milk as closely as possible. However, milk provides more than just the proper mix of nutrients in a readily ingested form. When human milk micelles are clotted by proteolytic enzymes under conditions simulating those found in the infant stomach, either no clot formed or the clots formed almost undetectable, very fine curds as opposed to the large curds formed by bovine milk (19). The large curds appear to slow gastric emptying (4, 19) and, therefore, the digestion time of the milk. The small clots may also be involved in the proper absorption of the milk constituents into the body for optimal utilization. These differences in the functional requirements of the casein micelle for the human digestive system are partially met by a micelle with a different composition and structure. The overall purpose of this research was to determine the relationships between structure and function of the casein micelles of human milk. Once these relationships are known, other characteristics of human milk may be matched in the preparation of substitutes for situations in which breast-feeding is not possible.

A large amount of the β-CN of bovine milk dissociates from the micelles upon cooling at 4°C (1, 2, 6, 18, 22, 29), probably because of the weakened hydrophobic bonds (17), leaving almost all of the α_{s1}-CN and α_{s2}-CN and most of the κ-CN intact. This dissociation of β-CN was dependent upon the time that the milk was cooled. The liberation of β-CN by cooling was also influenced by the size of the casein micelles (22). Large micelles released most of the β-CN, but the small micelles, which are rich in κ-CN, retained the β-CN but were the source of most of the released κ-CN. In that situation, the micelle sizes were essentially maintained. The release of β-CN leveled off when about half remained in the micelles. Conversely, removal of the calcium ions through dialysis or with EDTA (16, 20, 21, 27) caused dissociation of the

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micelles into smaller aggregates, suggesting that the α_s-CN associates with colloidal calcium phosphate largely through electrostatic interactions to form a framework for the micelles but that much of the β-CN is held in the micelles through hydrophobic interactions, which are weaker at low temperatures. This arrangement lends stability to the bovine casein micelle system and permits the delivery of the protein, calcium, and phosphate nutrients to the infant in a liquid suspension that is readily ingested. However, the human casein system contains only a very small amount of a protein corresponding to α_{s1}-CN (23). The majority of the caseins are in the β-CN fraction, which is unique in that it contains a single protein phosphorylated (**P**) at six levels from 0 (0-P) to 5 (5-P) (12, 13). Precipitation of this mixture by calcium ions is prevented by the stabilizing effects of the κ-CN in the formation of micelles. In this paper, the effects of cooling and the sequestration of calcium ions on the composition and size of human casein micelles are determined and related to the possible structure of the micelles.

MATERIALS AND METHODS

Fresh human milk obtained at >28 d postpartum was maintained at 37°C and skimmed in a prewarmed centrifuge (2300 × *g* for 10 min) according to the method of Sood et al. (26, 27). Phenylmethylsulfonyl fluoride (**PMSF**) was added to the skimmed milk to a final concentration of 0.2 m*M* to inhibit proteolytic activity.

Cooling Protocol and β-CN Composition of Micelles

Five-milliliter samples of the skimmed milk were cooled to 4°C for 0, 1, 6, 24, and 72 h. A portion of the 72-h sample was then incubated at 37°C for 3 h. Each sample was centrifuged at 110,000 × *g* for 1.5 h at 4°C to produce a pellet of casein micelles, which was then dissolved in the original volume of buffer A (4.5 *M* urea, 10 m*M* Tris, and 0.46 m*M* dithiothreitol; pH 8.0), and the total protein absorbance was determined at 280 nm (corrected for light scattering by measurements at 320 nm). To separate and quantify the different levels of the human β-CN relative to one another, samples and standards were analyzed on an FPLC® column (Mono Q HR5; LKB Pharmacia, Piscataway, NJ) using gradients between buffer A and buffer B (1.0 *M* NaCl in buffer A) as described by Dev et al. (8). The output from the FPLC® detector was fed into a computer and analyzed (3000 Series Chromatography Data System software; Nelson Analytical, Cupertino, CA) for peak areas. Samples of 0-P, 1-P, 2-P, 3-P, 4-P, and 5-P, purified as described earlier (14, 26), were used as standards.

Direct Addition of EDTA

To additional 10-ml samples of skimmed milk, maintained at 4 or 37°C, 50 m*M* EDTA (pH 7.2) was added by drop with constant stirring to yield final concentrations of 0, 1, 2, 3, 4, and 5 m*M* EDTA after water was added to give a final volume of 12 ml for each sample. The samples were then centrifuged to obtain pellets of the micelles, and these were treated and analyzed as described.

Addition of EDTA by Dialysis

Five-milliliter samples of skimmed milk that had been maintained at 4°C were dialyzed overnight against large excess volumes of simulated milk ultrafiltrate, prepared in deionized water as described by Sood et al. (28) to which PMSF (0.2 m*M* final concentration) and EDTA were added. The final concentrations of EDTA were 0, 2, 3, 4, and 5 m*M*. The pH of each sample was determined, and the samples were then centrifuged to obtain pellets of the micelles and analyzed as described. Because the milk was not stable for longer periods at 37°C, these studies were carried out only at 4°C.

Micelle Size and Voluminosity

Micelle size was determined by laser light scattering measurements that were performed on samples of skimmed milk at 37 and 4°C (Nicomp 370 Submicron Particle Sizer and Nicomp Analysis Software; Pacific Scientific, Silver Spring, MD). An Ostwald viscometer was used to determine the viscosity of skimmed milk samples at these two temperatures. In these cases, the amount of protein associated with the micelles was estimated from the difference in protein absorbance in the supernate after centrifugation of the micelles, using the assumption that most of the protein in the micelles was β-CN both before and after cooling (7, 8). The voluminosity of the micelles, which is the total volume per gram of material, was then calculated from those measurements using the equation of Eilers et al. (10). The amount of solvated water, in grams of H_2O per gram of protein, was calculated using the relationship described by Tanford (30) and an assumed mean partial specific volume for the protein of 0.70 ml/g.

RESULTS

Protein Loss from the Micelles

The striking changes in human milk that take place upon cooling are readily observed. Cooling to 4°C noticeably changes the opacity of the milk from a white, milky suspension to a translucent solution. This change is rapidly and completely reversed by warming to 37°C. Measurements of protein absorbance on the solubilized micelle pellets obtained before and after cooling of six milk samples showed that the initial loss of protein is rapid; 19.7 ± 7.5% ($\bar{X}$ ± SE) of the absorbance that is present at 37°C remains when the low temperature is attained. This value decreases to 13.8 ± 5.5% after 1 h, 11.8 ± 3.7% after 6 h, and 10.5 ± 5.5% as the 4°C temperature is maintained for 24 h. Protein absorbance then remains at that level up to 72 h (10.0 ± 4.4%). Reequilibration at 37°C for 3 h results in a return of most of the protein to the micelles so that 95.2 ± 15.7% of the original absorbance is present in the pellet.

β-CN Composition of Micelles

The separation of the micellar proteins on the Mono Q column and the identification of the various peaks by PAGE was described by Dev et al. (8). The completeness of the separation of the different forms β-CN is indicated by Figure 1A, which shows the Mono Q elution profile of a standard mixture of the highly purified forms of human β-CN that had been phosphorylated at six different levels. The positions of these six discrete peaks were used to identify the peaks found upon analysis of the micelle pellets. An example of an elution profile obtained after cooling is shown in Figure 1B. Integration of the six peak areas with the Nelson software, after assignment of a baseline to eliminate background and overlapping peaks, was translated into the relative proportions for the different β-CN in the micelles, expressed as percentages of the total β-CN composition in the pellet. The bar graphs of Figure 2 show these percentages for the control pellets at 37°C and for the remaining β-CN in the pellet after the specified interval of cooling at 4°C. In general, on prolonged cooling, the relative amount of 0-P in the micelles increased strikingly, 1-P increased less so, but 2-P, 3-P, 4-P, and 5-P decreased relative to 0-P and 1-P. These changes were reversed by incubation of the samples at 37°C for 3 h.

The total amount of calcium in human milk is about 8 m*M* of which 5 m*M* is colloidal. Addition of EDTA by drop at 4°C (Figure 3) showed changes that were similar to those caused by prolonged cooling, except that the decreases in the relative amounts of 3-P, 4-P, and 5-P were much greater as the EDTA concentration began to match the amount of ionized Ca^{2+}. Addition of EDTA by drop at 37°C (Figure 4),

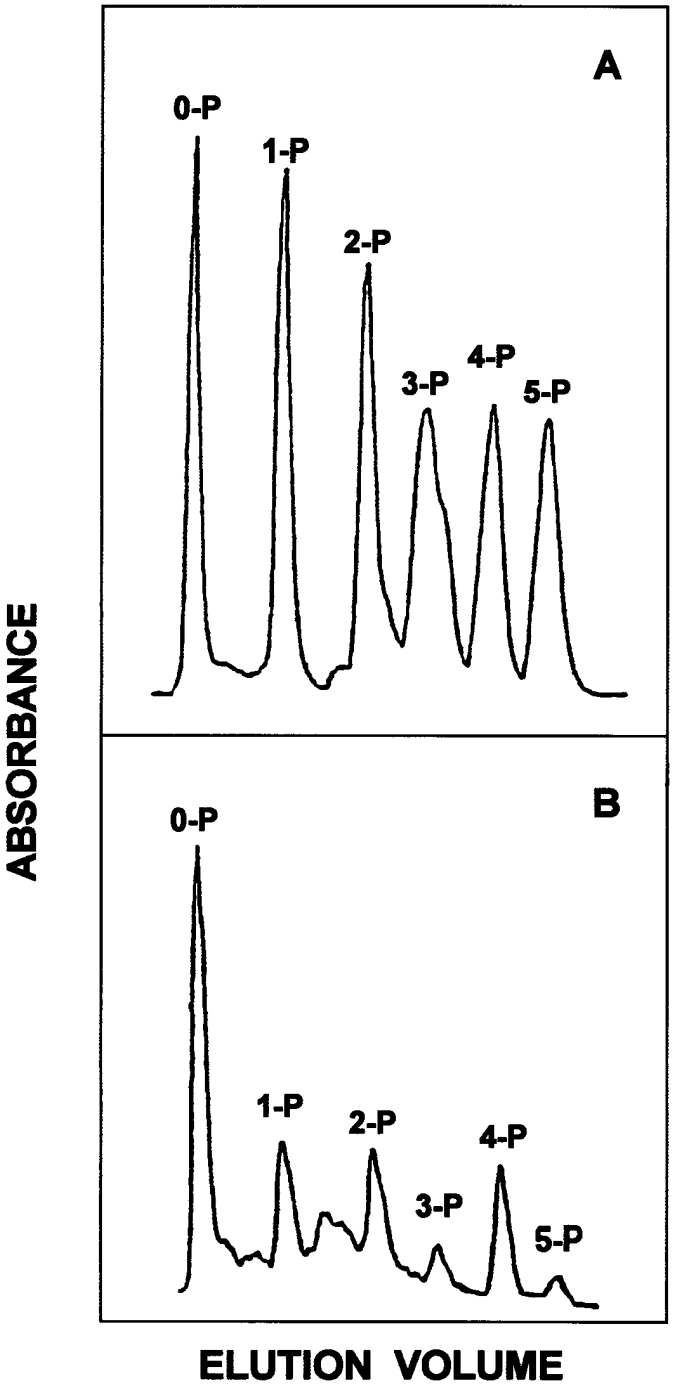

Figure 1. Elution profiles showing a standard mixture of the highly purified forms of human β-CN phosphorylated (P) at six different levels (A) and micelle pellet formed after cooling for 72 h (B) dissolved in buffer A and eluted with buffer B.

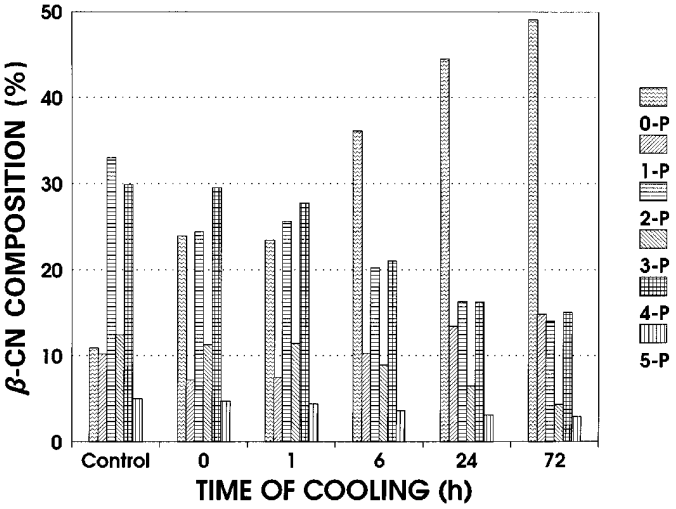

Figure 2. Relative percentages of the six different phosphorylated (P) forms of β-CN in the control pellet at 37°C and in the pellets after the specified intervals of cooling at 4°C.

when hydrophobic interactions were stronger, resulted in a significant change only at 7.5 m*M* EDTA concentration. At that point, the relative increase in 0-P was fairly large, and the 2-P and 4-P proteins decreased. The dialysis experiments to remove calcium yielded similar results. The sequestration of Ca^{2+} by EDTA caused the release of protons and a drop in pH to as low as 6.8 at the highest EDTA concentration, which could have enhanced micelle expansion and β-CN release. The investigation of pH effects on micelle dissociation is ongoing.

Mean Micelle Size and Solvation

Laser light scattering of the skimmed milk showed a broad range of size, but measurements may be approximately characterized by a mean Gaussian diameter in each case. The mean Gaussian diameter of the micelles in the samples was about 100 nm at 37°C and about 570 nm at 4°C. The original size was restored by rewarming the samples to 37°C for 3 h. However, rewarming for a much longer period led to an irreversible increase in particle size as the micelles aggregated, perhaps from proteolysis. The five- to sixfold increase in mean size upon cooling was accompanied by an increase in micelle solvation. Table 1 shows the voluminosity or volume in milliliter per gram of the micellar protein and its solvation in grams of H_2O per gram of protein at 4 and 37°C. Although the amount of protein associated with the micelles decreased with cooling, the volume increased because of water uptake.

Measurements of micelle sizes by laser light scattering after the addition of EDTA produced results that varied from the results for bovine milk. Rather than destabilizing the micelles and causing their dispersion to smaller sizes, removal of the calcium ions of human milk resulted in an increase in Gaussian diameter from 100 nm to about 150 nm for either dialysis or direct addition.

DISCUSSION

Previous studies with radioactively labeled κ-CN (8) indicated that some κ-CN is released, along with the β-CN, from human casein micelles upon cooling. These proteins are then pelleted with the micelles after reequilibration at 37°C for 3 h, and the original composition is attained. The reequilibrated protein particles in suspension also have size distribution and apparent voluminosity that are similar to those of the micelles that were not subjected to cooling, as indicated by calculations using laser light scattering and solvation. It seems likely that these proteins simply return to the original micelles rather than aggregating separately, as was suggested for the bovine system (5).

The Mono Q separation procedure for the casein pellets (8) was similar to that described by Kunz and Lönnerdal (15), except that their method did not separate the β-CN fraction into its six components. They (8) did, however, claim complete separation of the κ-CN and β-CN fractions. In the separation by Dev et al. (8), as used here, κ-CN overlapped the

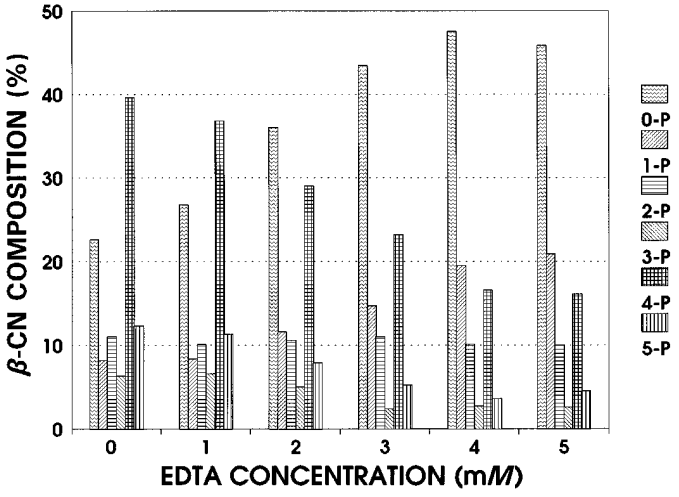

Figure 3. Relative percentages of the six different phosphorylated (P) forms of β-CN in micelle pellets after treating skim milk with varying EDTA concentrations at 4°C.

TABLE 1. Voluminosity and solvation of human casein micelles at different temperatures.

Temperature	Micellar protein	Relative viscosity	Voluminosity	Solvation[1]
(°C)	(g/100 ml)		(mg/g)	(g/g)
37	0.73	1.1459	7.18	6.48
4	0.68	1.1627	8.49	7.79

[1]Grams of H_2O per gram of micellar protein.

0-P and 1-P peaks and perhaps even extended into the 2-P fraction. Therefore, the peaks between these fractions in Figure 1B represent mostly κ-CN and show an increase in the proportion of κ-CN in the micelles after cooling. Because of the difference in extinction coefficients between κ-CN (7) and β-CN (26), some error may be present in the calculations of the amount of protein in the pellets and therefore in the calculated micelle voluminosity and solvation. However, an increased proportion of κ-CN and a higher extinction coefficient in the micelles after cooling would lead to an overestimation of the amount of protein and an underestimation of associated solvent. Thus, the real differences would be greater than those calculated here, and the general conclusions derived from the results relative to micelle structure should be valid.

The study results provide evidence that the release of β-CN from human micelles is similar to that reported for bovine casein micelles (5) in that the release depends on cooling time (9, 11, 24). However, the release of human β-CN is much more extensive. About 80% of the protein optical density was rapidly lost upon cooling to 4°C, most of which is β-CN according to the elution profiles. This loss may reflect the smaller size and much greater surface area of human micelles than of bovine micelles and suggests that human micellar β-CN is likely not shielded from the solution by κ-CN but has ready access to the surface and is held largely through hydrophobic interactions. After 24 h at 4°C, an additional 10% of the original protein, as indicated by absorbance at 280 nm, dissociated from the micelles, suggesting that some of the β-CN in the micelles is perhaps more internal but still only loosely associated. The remainder of the β-CN, which after 72 h is almost 50% 0-P and about 15% 1-P (Figure 2), is apparently more strongly self-associated or bound to κ-CN in the micelles. For bovine milk, α_{s1}-CN, which does not dissociate upon cooling, may play a structural role in the micelles as a framework upon which the β-CN may build (16). Rasmussen et al. (23) studied α_{s1}-CN in micelles of human milk obtained by ultracentrifugation after an unspecified time at 4°C and reported gel filtration peaks representing α_{s1}-CN, which constitute only about 10% of the total protein at most. This result probably indicates that the amount of α_{s1}-CN is less than any of the β-CN entities, except the 3-P and 5-P forms in the cooled micelles (Figure 2), and would be <1% of the total protein in the micelles under physiological conditions. It therefore seems unlikely that the α_{s1}-CN forms much of a micelle framework in the human system. These data suggest that the 0-P and 1-P forms of β-CN may function as such a framework instead. A loose structure for the micelles, indicated earlier by electron microscopy studies (3), is supported by the fact that the casein micelles of human milk, which are normally already more highly solvated than any other species [6.48 g of H_2O/g of protein compared with 3.35 for bovine (27)], actually increase in size and become more solvated, as revealed by laser light scattering and viscometric measurements after chilling at 4°C overnight (Table 1).

Analysis of treated bovine milks showed that increasing concentrations of EDTA can remove colloidal calcium and phosphate, thus increasing the concentration of serum casein (24) and causing disruption of the micelles. The results for human milk (Figures 3 and 4), which show an additional decrease in 3-P, 4-P, and 5-P upon calcium ion sequestration, indicate electrostatic interactions between these more highly phosphorylated molecules that contributed to the micelle stability. These interactions probably involve Ca^{2+} bridges between phosphoryl esters on adja-

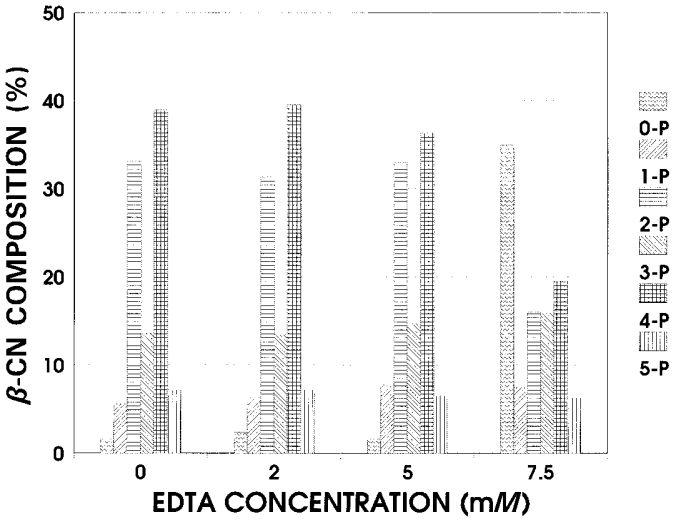

Figure 4. Relative percentages of the six different phosphorylated (P) forms of β-CN found in micelle pellets after treating skim milk with varying EDTA concentrations at 37°C.

cent molecules or through colloidal calcium phosphate. Similar suggestions have been made for bovine casein micelles for the α_{s1}-CN (5, 6, 31). The results of EDTA addition also indicate the presence of a very loose framework of mostly 0-P and 1-P β-CN, which is not changed when calcium ions are sequestered. This result also emphasizes the highly porous nature of the human casein micelles and lends support for a modified model that is similar to the one proposed by Slattery and Evard (25) for bovine micelle structure. Those data indicated that the micellar framework was probably still intact with the concentrations of EDTA that were added and that equilibrium exists such that β- and κ-CN, calcium, and phosphate may go back into the micelles under the proper conditions.

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REFERENCES

1 Ali, A. E., A. T. Andrews, and G. C. Cheeseman. 1980. Influence of storage of milk on casein distribution between the micellar and soluble phases and its relationship to cheese-making parameters. J. Dairy Res. 47:371.
2 Aoki, T., N. Yamada, and Y. Kako. 1990. Relation between colloidal calcium phosphate cross-linkage and release of β-casein from bovine casein micelles on cooling. Agric. Biol. Chem. 54:2287.
3 Carroll, R. J., J. J. Basch, J. G. Phillips, and H. M. Farrell, Jr. 1985. Ultrastructural and biochemical investigations of mature human milk. Food Microstruct. 4:323.
4 Cavell, B. 1979. Gastric emptying in preterm infants. Acta Paediatr. Scand. 68:725.
5 Creamer, L. K., G. P. Berry, and O. E. Mills. 1977. A study of the dissociation of β-casein from the bovine casein micelles at low temperature. N.Z. J. Dairy Sci. Technol. 12:58.
6 Davies, D. T., and A.J.R. Law. 1983. Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature. J. Dairy Res. 50: 67.
7 Dev, B. C., S. M. Sood, S. DeWind, and C. W. Slattery. 1993. Characterization of human κ-casein purified by FPLC. Prep. Biochem. 23:389.
8 Dev, B. C., S. M. Sood, S. DeWind, and C. W. Slattery. 1994. κ-Casein and β-caseins in human milk micelles: structural studies. Arch. Biochem. Biophys. 314:329.
9 Downey, W. K., and R. F. Murphy. 1970. The temperature-dependent dissociation of β-casein from bovine casein micelles and complexes. J. Dairy Res. 37:361.
10 Eilers, H., R.N.J. Saal, and M. Van der Waarden. 1947. Page 25 *in* Chemical and Physical Investigations on Dairy Products. Elsevier, Amsterdam, The Netherlands.
11 Fox, P. F., and J. Guiney. 1972. Partial fractionation of the α_s-casein complex. J. Dairy Res. 39:49.
12 Greenberg, R., and M. L. Groves. 1979. Human β-casein. J. Dairy Res. 46:235.
13 Groves, M. L., and G. Gordon. 1970. The major component of human casein: a protein phosphorylated at different levels. Arch. Biochem. Biophys. 140:47.
14 Javor, G. T., S. M. Sood, P. Chang, and C. W. Slattery. 1991. Interactions of triply phosphorylated human β-casein: fluorescence spectroscopy and light-scattering studies of conformation and self-association. Arch. Biochem. Biophys. 289:39.
15 Kunz, C., and B. Lönnerdal. 1990. Human-milk proteins: analysis of casein and casein subunits by anion-exchange chromatography, gel electrophoresis, and specific staining methods. Am. J. Clin. Nutr. 51:37.
16 Lin, S.H.C., S. L. Leong, R. K. Dewan, V. A. Bloomfield, and C. V. Morr. 1972. Effect of calcium ion on the structure of native bovine casein micelles. Biochemistry 11:1818.
17 Mahler, H. R., and E. H. Cordes. 1967. Page 111 *in* Biological Chemistry, Harper and Row Ltd., London, England.
18 Morr, C. V. 1975. Chemistry of milk proteins in food processing. J. Dairy Sci. 58:977.
19 Nakai, S., and E. L. Chan. 1987. Effect of clotting in stomachs of infants on protein digestibility of milk. Food Microstruct. 6: 161.
20 Ono, T., H. T. Dan, and S. Odagiri. 1978. Dissociation of bovine casein micelles by dialysis. Agric. Biol. Chem. 42:1063.
21 Ono, T., T. Furuyama, and S. Odagiri. 1981. Dissociation of large and small bovine casein micelles by dialysis. Agric. Biol. Chem. 45:511.
22 Ono, T., T. Murayama, S. Kaketa, and S. Odagiri. 1990. Changes in protein composition and size distribution of bovine casein micelles induced by cooling. Agric. Biol. Chem. 54:1385.
23 Rasmussen, L. K., H. A. Due, and T. E. Petersen. 1995. Human α_{s1}-casein: purification and characterization. Comp. Biochem. Physiol. 111B:75.
24 Rose, D. 1968. Relation between micellar and serum casein in bovine milk. J. Dairy Sci. 51:1897.
25 Slattery, C. W., and R. Evard. 1973. A model for the formation and structure of casein micelles from subunits of variable composition. Biochim. Biophys. Acta 317:529.
26 Sood, S. M., P. Chang, and C. W. Slattery. 1985. Interactions in human casein systems: self-association of fully phosphorylated human β-casein. Arch. Biochem. Biophys. 242:355.
27 Sood, S. M., D. K. Gaind, and R. K. Dewan. 1979. Correlation between micelle solvation and calcium content. N.Z. J. Dairy Sci. Technol. 14:32.
28 Sood, S. M., G. Javor, K. Kjoller, V. Ellings, and C. W. Slattery. 1992. Bovine and human casein micelles as seen by scanning tunneling/atomic force microscopy. FASEB J. 6:A116.
29 Sood, S. M., K. S. Sidhu, and R. K. Dewan. 1977. Voluminosity of casein micelles in chilled milk from buffalo and cow. Indian J. Dairy Sci. 30:7.
30 Tanford, C. 1955. Intrinsic viscosity and kinematic viscosity. J. Phys. Chem. 59:798.
31 Watanabe, M., I. Katou, K. Shimazaki, R. Niki, and S. Arima. 1973. Effects of temperature and urea on casein micelle components. Jpn. J. Zootech. Sci. 44:148.