Effect of Calcium Ion on the Structure of Native Bovine Casein Micelles†

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ABSTRACT: The effect of Ca^{2+} activity on the sedimentation velocity in a sucrose gradient and the hydrodynamic radius (measured by inelastic laser light scattering) of fractionated native casein micelles has been studied. Removal of subcritical amounts of Ca^{2+} from casein micelles with EDTA releases soluble casein and results in a progressive decrease in the sedimentation velocities of the micelles without altering their hydrodynamic radii. These findings suggest that Ca^{2+} removal initially dissociates weakly bound caseins from the micelle, while a size-determining micellar framework remains intact. Starch gel electrophoresis indicates that the framework protein is largely α_s-casein, and the dissociable protein is mainly β- and κ-casein. When Ca^{2+} activity falls below a critical level, the micelles completely dissociate and no evidence of the micellar framework is found. Addition of small amounts of Ca^{2+} to native casein micelles eliminates the soluble casein and increases the sedimentation velocities of the micelles without increasing their hydrodynamic radii, suggesting that soluble caseins can be incorporated within the interstices of the micellar framework.

The structure and size characteristics of native casein micelles have been the subject of many recent studies (Shimmin and Hill, 1965; Waugh and Noble, 1965; Payens, 1966; Rose and Colvin, 1966; Morr, 1967; Parry and Carroll, 1969; Rose, 1969; Ribadeau Dumas and Garnier, 1970; Schmidt and Buchheim, 1970; Lin *et al.*, 1971); however, no generally accepted model of the micelle has emerged. Skim milk contains about 3.2 mmoles/100 ml of total calcium of which about one-third is in the dissolved state and the balance is associated with casein micelles and phosphate in the colloidal state (Jenness and Patton, 1959). The Ca^{2+} concentration is about 2.5–3.4 mM (Tessier and Rose, 1958). It is clear that calcium, both in ionic and colloidal forms, plays a critical role in stabilizing micellar casein structure (Ford *et al.*, 1955; von Hippel and Waugh, 1955; Bohren and Wenner, 1961; Rose, 1968; Morr *et al.*, 1971b). Removal of Ca^{2+} with EDTA and other complexing agents causes dissociation of casein micelles, and addition of excess Ca^{2+} favors their aggregation. In this paper we report the effect of controlled removal and addition of Ca^{2+} on casein micelle size as determined by inelastic light scattering and rate-zone ultracentrifugation in sucrose gradients, and discuss the implication of these findings for a model of micelle structure.

Materials and Methods

Native skim milk casein micelles were separated into different sized fractions by rate-zone ultracentrifugation using sucrose gradients prepared in lactose-free simulated milk ultrafiltrate (SMUF)[1] (Jenness and Koops, 1962) as previously described (Morr *et al.*, 1971a; Lin *et al.*, 1971). In this study we used two different micelle fractions, *i.e.*, gradient fractions 4 and 6 obtained by sedimentation at 30,000 rpm for 20 min (Lin *et al.*, 1971). These fractions, which contained about 1.5 mg of casein/ml, were previously found to be essentially monodisperse by electron microscopy and by inelastic light scattering and to have hydrodynamic radii of about 560 Å (fraction 4) and 650 Å (fraction 6) after removal of sucrose by dialysis against SMUF.

In initial experiments calcium was progressively removed from the micelle fractions by direct dropwise addition of 0.032 M EDTA. In later experiments reported here, calcium removal was more carefully controlled by dialysis of the micelle fractions for one day at 5° against SMUF to which various amounts of 0.16 M EDTA had been added (Table I). The EDTA solution was prepared from Na_2EDTA–Na_4EDTA (5:8, w/w) so that its addition would not change the pH (6.6) of SMUF. Addition of Ca^{2+} to micelles was carried out in a similar manner by dialyzing the micelle fraction against SMUF which contained various amounts of 0.5 M $CaCl_2$. Changes in Ca^{2+} activity in the micelle fractions were measured with an Orion Ca^{2+} electrode using SMUF as a reference, and are reported here directly in millivolt differences from that of SMUF (Table I).

Distribution of casein species in the micelle fractions was determined by starch gel electrophoresis (Morr, 1971).

Hydrodynamic radii, R, were obtained using the inelastic laser light-scattering apparatus with homodyne beat detection described previously (Lin *et al.*, 1971). The radius is inversely proportional to the half-width of the lorentzian curve which describes the broadening of the incident laser frequency. The experimental scattering curves were accurately described by single lorentzians, and the estimated precision of R is $\pm 2\%$.

All measurements and procedures were carried out at pH 6.6 and 20–25° unless otherwise indicated.

Results

To determine the effect of Ca^{2+} activity on the sedimentation behavior of casein micelles, micelle fractions were dialyzed against SMUF containing EDTA or added Ca^{2+} in the concentrations indicated in Table I. An 0.5-ml aliquot of each

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[1] Abbreviation used is: SMUF, simulated milk ultrafiltrate.

TABLE I: Effect of Altering Ca^{2+} Activity on Hydrodynamic Radius and Inelastic Light-Scattering Intensity of Casein Micelles.

Dialysis Condn	mmole/100 ml EDTA[c]	mmole/100 ml Ca^{2+} [d]	$E_{Ca^{2+}}$ [e] (mV)	PMT[f] (Voltage, V)	Radius (Å) Fraction 4[g]	Radius (Å) Fraction 6
1[a]			0	1150	560	650
2	0.016		−1.0	1170	560	640
3	0.032		−1.3	1200	560	630
4	0.056		−2.0	1230	560	640
5	0.080		−2.7	1260	560	
6	0.12		−3.6	1400	560	650
7[b]	0.90		−20.0			
8		0.25	5.0	1200	560	630
9		0.50	6.0	1200	600	630

[a] Original SMUF, pH 6.6, total [Ca] = 0.9 mmole/100 ml. [b] Added EDTA was equivalent to Ca in SMUF. Micelles were completely disrupted. [c] Amount of EDTA (0.16 M) added per 100 ml of SMUF. [d] Amount of Ca (0.5 M $CaCl_2$) added per 100 ml of SMUF. [e] Difference in Ca^{2+} electrode potential measured with Orion Ca^{2+} electrode at 20° after completion of dialysis, using SMUF as reference. [f] Photomultiplier voltage needed to obtain a scattering signal of standard strength. High voltages mean low scattering intensities. [g] A single half-width (470 Hz) corresponding to radius of 560 Å was found to describe all the experimental lorentzian curves except that of condition 9. Estimated uncertainties of half-widths and radii are ±2%.

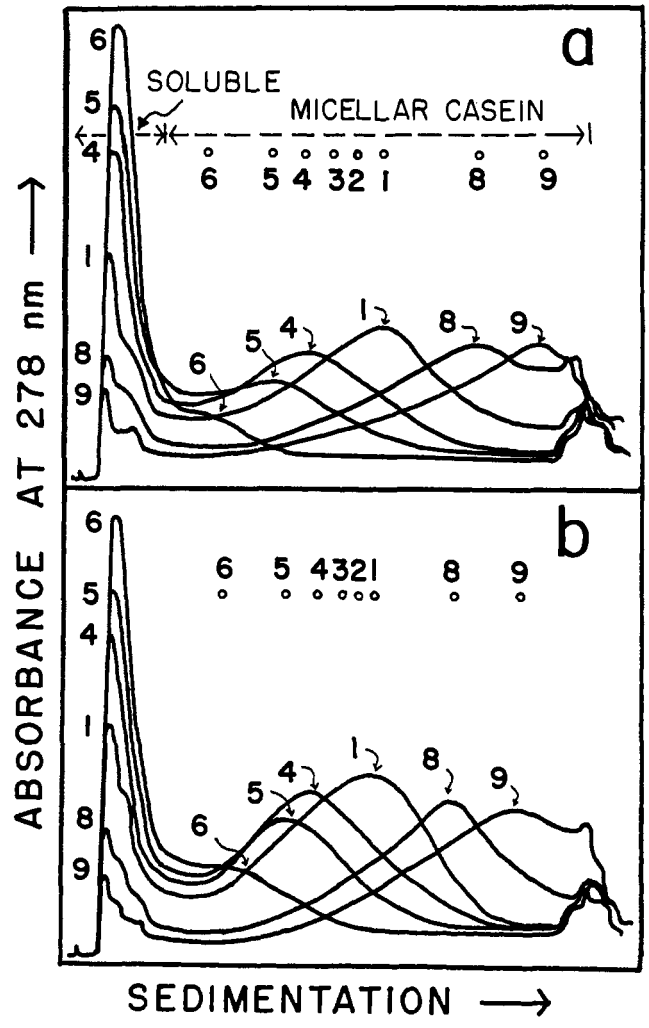

FIGURE 1: Distribution of casein in a 15–25% sucrose gradient after sedimentation for 20 min: (a) at 35,000 rpm for casein micelle fraction 4 and (b) at 30,000 rpm for casein micelle fraction 6. Curves are numbered according to dialysis conditions specified in Table I. Although curves 2 and 3, which were located between curves 1 and 4, and curve 7, which contained only a soluble casein peak positioned near the meniscus, are not shown here, the position of all the micelle peaks is indicated by the numbered open circles above the curves.

dialyzed micelle fraction was layered on a 15–25% sucrose gradient, and centrifuged for 20 min at 30,000 or 35,000 rpm. Sedimentation patterns, obtained by monitoring the gradient at 278 nm (Morr *et al.*, 1971a), are shown in Figure 1a,b for fractions 4 and 6, respectively. Each sedimentation pattern consisted of a broad micelle peak located in the middle of the gradient and a soluble casein peak sedimenting near the meniscus. Reducing the Ca^{2+} activity of both micelle fractions caused a shift of the broad micelle peak toward the meniscus, accompanied by a concomitant transfer of casein from the micelle peak to the soluble casein peak (curves 1–6).

A critical point in Ca^{2+} activity reduction was observed (condition 6 in Table I), beyond which the micelle peak in Figure 1a,b disappeared. At this point, the EDTA addition was equivalent to only 13% of the total Ca in SMUF as compared to that of condition 7 in Table I.

Inelastic light-scattering studies of the dialyzed micelle fraction gave the results summarized in Table I. Up to the critical point of Ca^{2+} activity reduction (condition 6 in Table I), the hydrodynamic radius remained constant for each micelle fraction; however, the increased photomultiplier voltage necessary to detect the scattering indicates that the amount of high molecular weight scattering material (micelles) was decreasing. Beyond this critical point, the light-scattering spectrum became very weak and nonlorentzian, indicating the abrupt disappearance of large, monodisperse scattering particles.

In our earliest experiment where EDTA was added dropwise with stirring rather than by dialysis, the spectral half-width remained constant as above but no shift of the broad micelle peak in sedimentation was observed, although the amount of micellar casein decreased. These observations indicate that some of the micelles were completely dissociated while the rest of them still remained intact.

We also indicate in Figure 1a,b and Table I the results of adding Ca^{2+} to micelles in SMUF by dialysis. Addition of small amounts of Ca^{2+} caused the micelles to sediment at a faster rate, along with the transfer of soluble casein to the micelle sedimentation peak (curve 8 in Figure 1a,b), without altering their hydrodynamic radii (condition 8 in Table I). Further addition of Ca^{2+} increased the sedimentation rates of both fractions (curve 9 in Figure 1a,b) and the hydrodynamic radius of fraction 4 (condition 9 in Table I), indicating formation of progressively larger casein particles. Direct addition of small amounts of Ca^{2+} to micelles in SMUF also yielded faster sedimenting micelles with larger hydrodynamic radii. However, further addition of Ca^{2+} by this procedure caused gross aggregation and precipitation of the micelles.

Casein micelles are composed of three major types of proteins: α_s-, β-, and κ-caseins (Thompson *et al.*, 1965). To

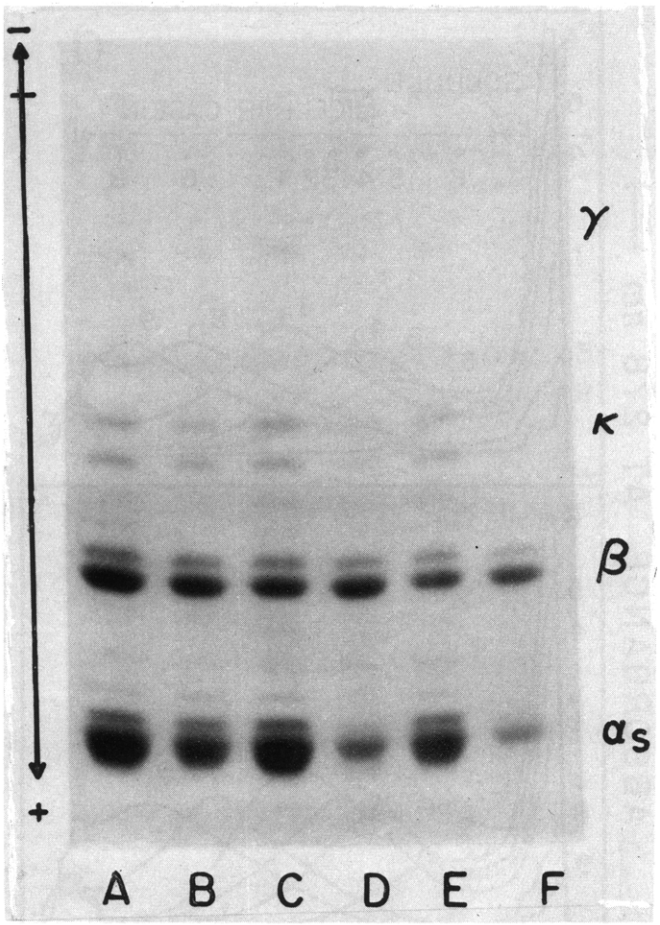

FIGURE 2: Starch gel electrophoresis patterns of casein species in: (A and B) total casein micelle fractions 6 and 4, respectively; (C and D) micellar and soluble portions of micelle fraction 6; and (E and F) micellar and soluble portions of micelle fraction 4. All micellar and soluble portions (C–F) were obtained by dialysis of micelle fractions 6 and 4 *vs.* SMUF containing 0.016 mmole of EDTA/100 ml (condition 2 in Table I) and centrifugation at 30,000 rpm for 20 min as in Figure 1.

determine which of these, if any, is preferentially dissociated from the micelles by dialysis against SMUF containing EDTA or removal of Ca^{2+}, we compared the starch gel electrophoresis patterns of the proteins obtained from the original casein micelle fractions, and those collected from the broad micelle and soluble casein peaks shown in Figure 1. The results are shown in Figure 2. Most of α_s-casein remained with the micelle casein, whereas the soluble casein was predominantly β- and κ-caseins.

Discussion

The above results enable us to construct a model for the structure and dissociation of casein micelles. The sedimentation velocity of the micelles decreases as Ca^{2+} activity is reduced. This might be attributed either to a decrease in micelle molecular weight; an increase in solvated partial specific volume, leading to a decrease in the buoyancy factor $(1 - \bar{v}\rho)$; or an increase in hydrodynamic radius, leading to an increase in frictional coefficient. The inelastic light-scattering measurements show, however, that the hydrodynamic radius of the predominant large scattering species, *i.e.*, micelles, remains essentially constant as long as the Ca^{2+} activity remains above a critical value. However, the scattering intensity decreases as Ca^{2+} activity is reduced, indicating a decrease either in molecular weight or in the concentration of micelles, or both. It is highly unlikely that removal of Ca^{2+} changes the osmotic conditions of the solution enough to alter markedly the buoyancy factor. Thus, the decrease in sedimentation velocity is most likely due to a decrease in micelle molecular weight. Rate-zone ultracentrifugation and starch gel electrophoresis results substantiate that these treatments cause dissociation of caseins from the micelles and indicate that it is predominantly β- and κ-caseins which dissociate from the micelle upon removal of Ca^{2+}. It therefore appears plausible to envisage casein micelles as composed of a size-determining micellar framework, which is predominantly α_s-casein and colloidal phosphate, with β- and κ-caseins attached to the framework and filling its interstices through Ca^{2+} bridges. Removal of Ca^{2+} in small amounts leads to dissociation of the relatively weak bonds holding β- and κ-caseins in the micelle, but the remaining framework still has the same frictional resistance as the intact micelle. (It has been shown both theoretically (Bloomfield *et al.*, 1967) and experimentally with viruses and macroscopic models (Douthart and Bloomfield, 1968) that packed and porous structures of the same size have virtually identical frictional coefficients.) When a critical amount of Ca^{2+} is removed the micellar framework also dissociates.

These results appear to be in accord with those discussed by Waugh (1971) with regard to the "cooperative" or "all-or-none" influence of Ca^{2+} on the stability of casein micelles. The ionic calcium concentration in skim milk is close to 0.002 M. However, Waugh (1971) has shown that the dialysis of first cycle caseins against a buffer with Ca^{2+} as low as 0.0011 M, containing the proper concentrations of phosphate, citrate, and sodium ions, results in the formation of micelles which have the same size distribution as those of skim milk, as judged by differential centrifugation. Thus both with regard to dissociation of micelles by removal of Ca^{2+}, or re-formation of micelles by addition of Ca^{2+} to an appropriate buffer system, there appears to be a range of Ca^{2+} concentrations, below that characteristic of skim milk, over which the micellar framework is stable or can be formed. Below a critical Ca^{2+} concentration, the framework dissociates abruptly, and reassociation is impossible.

Casein micelles can be considered as porous particles (Ribadeau Dumas and Garnier, 1970) containing Ca^{2+} bound at carboxyl groups and associated with colloidal phosphate (Yamauchi *et al.*, 1969). It has been shown by earlier workers that removal or addition of Ca^{2+} to milk alters the sedimentation characteristics of casein micelles (von Hippel and Waugh, 1955; Bohren and Wenner, 1961). It is therefore reasonable that these treatments may control the size and/or weight of the micelles.

Observations made in the present study indicate that there may be two types of calcium linkages which stabilize native micelle structure, in agreement with the work of Manson (1962). One type, the stronger, may be primarily responsible for formation and stability of the micellar framework, while the other may serve as Ca^{2+} bridges between the framework and soluble caseins. Yamauchi *et al.* (1969) observed a "hard-to-exchange calcium" associated with the colloidal phosphate of casein micelles in milk, a "slowly exchangeable calcium" in colloidal phosphate-free milk, and a "readily exchangeable calcium" in composite caseinate dispersion. It is possible that initial removal of Ca^{2+} from casein micelles by addition of low levels of EDTA causes dissociation of soluble caseins (mainly β- and κ-caseins) from the framework by disrupting the easily exchangeable Ca^{2+} bridges. Further

removal of Ca^{2+} from casein micelles with higher concentrations of EDTA dissociates the colloidal calcium phosphate and Ca-α_s-caseinate bonds, and thereby destroys the micellar framework. This picture accords with the results of Snow and Hay (1966).

In further support of this model for the structure and mechanism of dissociation of casein micelles, it should be noted that β-casein has been found to dissociate readily and reversibly from casein micelles in the cold (Rose, 1968). Downey and Murphy (1970) have also observed that the removal of most (approximately 60%) of the β-casein from casein micelles of milk by gel filtration at 5° on Sepharose 2B columns is not attended by a marked decrease in the size of the casein micelles, nor is there any indication of micellar disintegration. The remainder of the β-casein (approximately 40%) appears to be more strongly bound to the α_s- and κ-caseins and may be involved in the internal cohesion of casein micelles. It follows that most (~60%) of the β-casein appears to be loosely associated with the casein micelles of milk, from which it dissociates as the Ca^{2+} activity is reduced. This model is consistent with the proposition (Payens, 1966; Rose, 1969) that β-casein plays a structural role within casein micelles, if the qualification is made that less than half of the total β-casein is sufficient for this function.

The results obtained upon direct dropwise addition of EDTA to the micelles in SMUF may be explained by supposing that a high local concentration of EDTA lowers the Ca^{2+} ion activity below the critical value, thus leading to complete dissociation of the micellar framework in the vicinity of the EDTA droplet. Since the casein solutions were thoroughly mixed by swirling them during addition of each EDTA droplet, it may be inferred that micelle framework dissociation is a rapid process, occurring in a few seconds or less.

The results obtained upon addition of Ca^{2+} to micelles in SMUF may also be explained in terms of the micellar framework model. Incorporation of soluble casein into micelles, as shown above, takes place without changing the hydrodynamic radius of the micelles, indicating that the micellar framework can accept additional soluble caseins within its interstices. Addition of still larger amounts of Ca^{2+} apparently leads to aggregation of the micelles, as evidenced by the initial increase in the micellar hydrodynamic radii, followed by their eventual precipitation.

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