Effect of the Anion Citrate on the Mineral Composition of Artificial Casein Micelles

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The composition of artificial casein micelles (ACM) prepared at constant concentration of caseins, calcium (Ca), and phosphate (P_i) in media with different citrate (Cit) concentrations was studied. The incorporation of the different mineral and protein components to the ACM was conditioned by the Cit concentration. In our working condition, the ACM size remained almost constant for Cit concentration ranging from 7 to 10 mM. This behavior could be indicating that the action of Cit esentially consists of a regulation of the Ca activity. The molar ratio at which Ca and P_i were incorporated to the ACM varied for different Cit concentrations. At decreasing pH, the Ca/P_i molar ratios for the remanent ions in the ACM were dependent on the Cit concentration. These observations could be related to a certain kind of competition between Cit, micellar calcium phosphate (MCP), and other groups able to bind Ca in the ACM.

Keywords: *Artificial casein micelles; citrate; mineral composition*

INTRODUCTION

Casein micelles (CM) are almost spherical casein agregates of 20−600 nm in diameter, highly hydrated and stabilized as colloidal suspension in milk. Their major casein constituents are α_{S1}-, α_{S2}-, β-, and κ-casein at a molar ratio of about 4:1:4:1.3. CM also contain about 7% inorganic materials, predominantly constituted by micellar calcium phosphate (MCP), also referred to as colloidal calcium phosphate, which plays an important role in maintaining the CM structure (Walstra, 1990). In effect, MCP removal produces partial dissociation of caseins from CM, suggesting that MCP fulfills a cross-linking function (Dalgleish and Law, 1989; Visser et al., 1979). CM also contain other milk minerals ions such as Mg and citrate (Cit), and although several models have been proposed, the precise structure of the CM colloidal mineral and their interactions with caseins have not yet been fully elucidated (McMahon and Brown, 1984; Aoki et al., 1996). Particularly in the case of Cit, although it is generally accepted that its presence in the CM is related to the formation of MCP, the participation of this anion in the CM structure still remains unknown (van Dijk, 1990a).

ACM systems were frequently used to study the interaction of Ca, Mg, P_i, and Cit ions with caseins (Aoki, 1965; Schmidt, 1979). These micellar systems, which can be prepared with different caseins and salt composition, are useful in the study of the effect of the separate constituents on micellar properties (Schmidt, 1979).

In the present work, the mineral and protein composition of ACM obtained with different Cit concentration and pH values were examined in order to obtain further insight into the role of Cit in the micellar structure.

MATERIALS AND METHODS

Preparation of ACM. ACM were prepared according to the method of Knoop et al. (1979) with minor modifications. Whole bovine casein was prepared from suspensions of commercial, nonfat dried milk (MOLICO, Société des Produits Nestlé S.A., Vevey, Switzerland) reconstituted to 10% (w/v) in distilled water by acid precipitation at pH 4.6 with 1 M HCl. The precipitated casein was washed several times with distilled water and dissolved in distilled water at room temperatures (23−25 °C) at a concentration of 28 g/L by gradual addition of 1 M NaOH, ensuring that the pH did not exceed 7.0. The pH was finally adjusted to 6.8. The ACM were formed at room temperature by adding to the casein solution the amount of 1 M sodium citrate necessary to reach the desired final Cit concentration, with 15-min stirring. The prescribed Ca and P_i final concentrations were obtained by five additions of 0.2 M calcium chloride and 0.2 M dipotasium acid phosphate with continuous stirring, with 15-min intervals between them.

The volume and the pH were adjusted in order to reach a final casein concentration of 14 g/L and a final pH of 6.8. The final concentrations of Ca and P_i were 25 and 20 mM, respectively. The final Cit concentrations varied from 5 to 15 mM. Sodium azide was added at the rate of 0.05% (w/v) to ACM as a preservative.

ACM at different pH values were prepared by adjusting the pH of a sample of the original suspension by slow addition of 3 M HCl and vigorous stirring. After 24-h storage at 4 °C, the samples were heated to room temperature, and their pH was measured after 30-min equilibration. Small pH corrections were sometimes necessary to achieve the desired value.

Composition of the Colloidal Phase. The ACM formed were separated at 25 °C by centrifugation at 250000*g* for 90 min, using a Beckman L8-80M ultracentrifuge. The amounts of the different components in the colloidal phase were calculated from the difference between the total concentrations and the concentrations in the supernatants. Diffusible ions were also determined in the ultrafiltrates obtained from several samples to estimate their binding to free caseins. These ultrafiltrates were prepared by centrifugation at 1200*g* for 30 min of 15 mL of the ACM suspension in a loop of dialysis tubing suspended in a 50-mL conical centrifuge tube at room temperature.

Size Variations of the ACM. The changes in the ACM size were followed by the wavelength (λ) dependence of turbidity (τ) of the suspensions, measured as $\alpha = -d(\log \tau)/$

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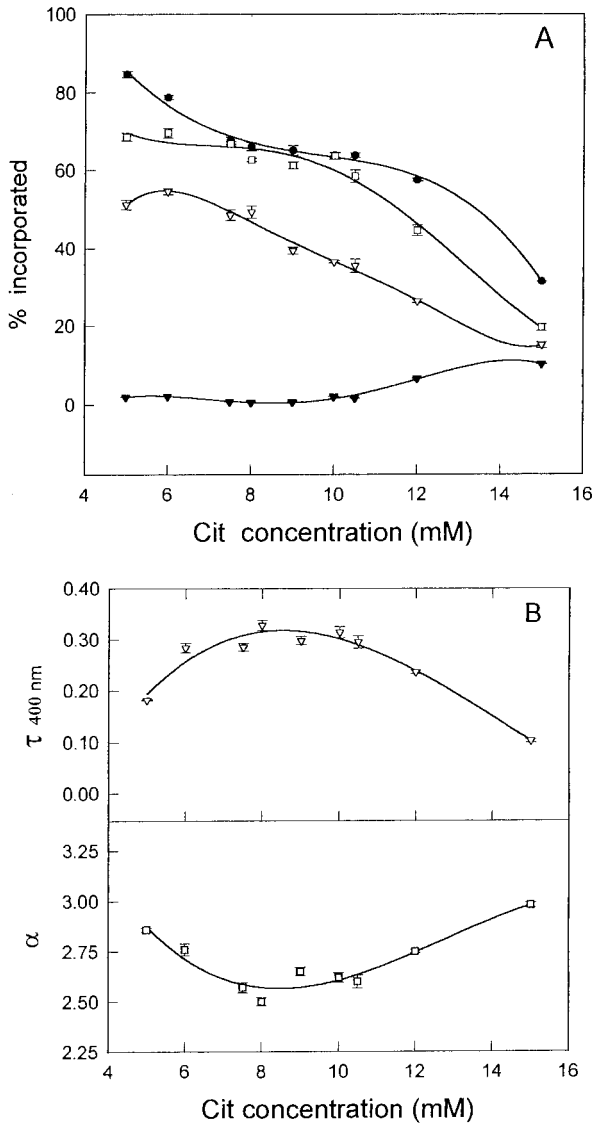

Figure 1. (A) Fractions of the total calcium (□), inorganic phosphate (▽), citrate (▼), and caseins (●) incorporated to the ACM expressed as percentage. (B) Turbidity (τ) (▽) and $\alpha = -d(\log \tau)/d(\log \lambda)$ (□) for ACM prepared at pH 6.8 and variable Cit concentration. Final concentration: Ca 25 mM, P_i 20 mM, and casein 14 g/L. Temperature: 23−25 °C. Each experimental point is the average (±SE) of at least three determinations.

$d(\log \lambda)$ (Horne, 1986). α was obtained from the slope of log τ vs log λ plots in the 400−600-nm range.

Determination of Caseins, Ca, P_i, and Cit Concentrations. Casein concentration was determined according to Kuaye's method (1994). Ca was determined by atomic absorption spectrometry (Metrolab RC 250 AA), and P_i was determined by a standard colorimetric method. Cit was determined by the colorimetric method of Marier and Boulet using TCA filtrates as described by White and Davies (1963).

RESULTS AND DISCUSSION

Figure 1A shows the minerals and the casein incorporated to ACM prepared with different Cit concentrations. Casein, Ca, and P_i total concentrations in the final ACM preparation were similar to those of milk, and the pH was maintained at 6.8. The values of α for each of the preparations (Figure 1B) showed that the biggest particles were obtained with Cit concentrations ranging from 7 to 10 mM. Smaller particles were formed with Cit concentrations either lower or higher than this concentration range.

Although the incorporation of caseins to the ACM decreased for increasing Cit concentrations, it remained almost constant in the Cit concentration range mentioned above. This was also the case for the incorporated Ca but not for the incorporated P_i, which decreased in all the range of increasing Cit concentration used. The Cit incorporated to the ACM was almost null until an external Cit concentration of about 11 mM was reached, starting then to increase with increasing Cit concentration in the medium. The amounts of ions retained by the total protein fraction, ACM, and free caseins obtained by ultrafiltration at 8, 10, and 12 mM Cit did not show significant differences with the amounts incorporated by the ACM obtained by sedimentation of these particles at the same Cit concentration, showing that in this range of Cit concentrations the interaction of the ions with free caseins was negligible.

The behavior described above suggested that the presence of Cit in the medium was related to the progressively lower participation of P_i in the ACM structure. This effect could be associated with the complexing of Ca by Cit with a decrease of the activity of the cation in solution. This activity decrease could shift the equilibrium of MCP formation to free ions production. It must be noted at this regard that, at the pH value here used, for Cit concentrations lower than the Ca concentration, more than 95% of the anion is bound in a 1:1 complex with Ca, thus reducing the effective Ca level by this amount (Horne, 1982). Until 10 mM Cit, however, the amount of Ca incorporated to the ACM remained almost constant, a fact perhaps related to the diferent kinds of groups that can bind Ca in the CM structure. In effect, experimental evidence presented by different authors suggested that Ca, besides its participation in the MCP, could be directly bound to negatively charged groups of the caseins (e.g., carboxyl groups) or to other casein residues as part of complexes that do not participate in the MCP structure (van Dijk, 1992). Another point is that the decrease of the P_i incorporated to the ACM in the range of Cit concentrations from 7 to 10 mM was not accompanied by changes in ACM size. It has been shown for CM that a fraction of its MCP can be dissociated by acidificaction without any change in CM size. This result suggests the existence of a micellar frame that needs only a part of the total MCP to be held (de Kruif and Roefs, 1996). In a similar way, following the increase in size of α_{S1} casein−P_i aggregates by changes in α, Horne (1982) has found a certain evidence suggesting that the size of the particles was fixed very early in the reaction as an open network, and as the reaction progressed an infilling of the interstices gradually occurred. Although Horne's results referred to the α_{S1} casein alone, the fact that this is the major protein component of the CM allows us to consider that a similar behavior could be possible in the precipitation of the mixture of caseinates. The presence of Cit in concentrations from 7 to 10 mM could then progressively inhibit the formation of the MCP fraction not necessary to maintain this micellar frame, producing also the relocation of Ca on other binding sities. In a different approach, taking into account the model proposed by van Dijk for MCP formation as a chemical reaction better than a solid phase separation, it is possible to think that the amount of MCP formed and the size of the agregates obtained were kinetically controlled (van Dijk, 1990b). MCP formation could then appear as a chemical reaction competing with other

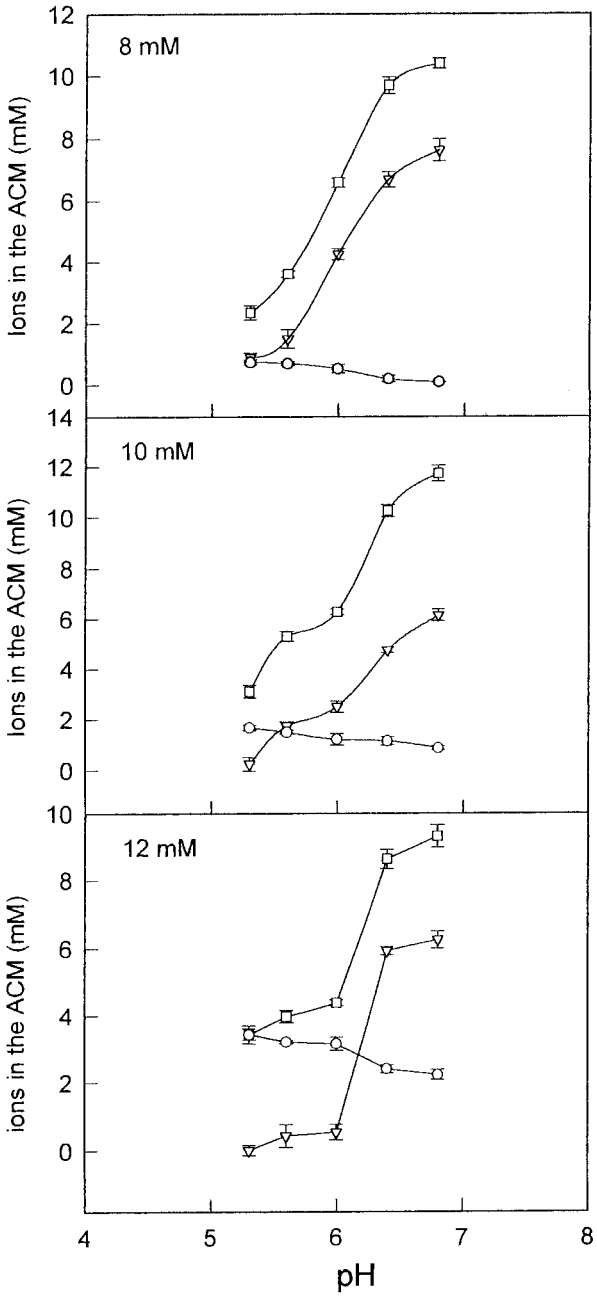

Figure 2. Concentration of calcium (□), inorganic phosphate (▽), and citrate (○) in ACM prepared at 8, 10, and 12 mM Cit and acidified to different pH values. Temperature: 23−25 °C. Each experimental point is the average (±SE) of at least three determinations.

kinds of interactions involving Ca and the caseins, and Cit could affect this complex equilibrium by its complexing action on Ca. Results reported by Horne (1982) showed that the role of Cit in the precipitation of α_{S1} casein from casein−Ca−Cit mixtures appeared to be merely the subtraction of the free cation from the medium, without any active participation in the mechanism of the precipitation. At concentration over 10 mM, the action of Cit started to inhibit the Ca and the caseins uptake by the micellar structure, producing a decrease in the amount and size of the ACM formed and an increasing uptake of Cit.

Figure 2 shows the remanent Ca, P_i, and Cit in the ACM acidified to different pH values as functions of the pH of the medium. The amount of the remanent ions was calculated as the difference between their initial

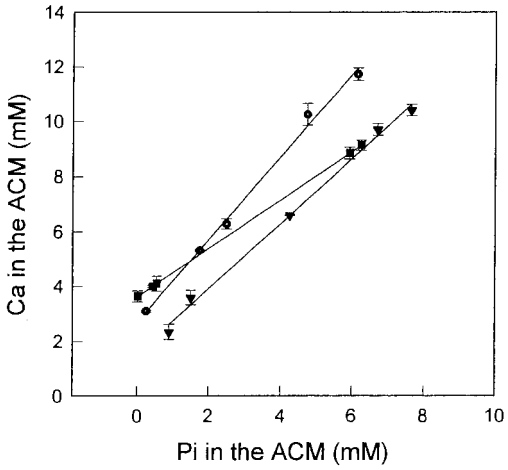

Figure 3. Concentrations of Ca and inorganic phosphate in the ACM at different pH values. Citrate concentrations: (▼) 8, (●) 10, and (■) 12 mM. Temperature: 23−25 °C. Each experimental point is the average (±SE) of at least three determinations.

concentrations and the concentrations in the supernatants of the sedimentation at 250000*g* for 90 min. Ca and P_i decreased with decreasing pH, while Cit showed increasing incorporation to ACM. Plotting the Ca remanent in the ACM as a function of the remanent P_i at different pH (Figure 3), straight lines were obtained for the different Cit concentrations used. Interpreting these plots in a similar way as the plot obtained for the acid dissociation of the mineral fraction of CM by van Hooydonk et al. (1986), the intercept of such lines could be taken as a measure of the bound Ca not integrated to the MCP structure, and their slopes could give us the Ca/P_i molar ratio in the MCP formed, provided that the fraction of Ca not integrated to the MCP remained constant at the different pH values used. The slopes obtained at different Cit concentrations were different, suggesting either that the Ca/P_i molar ratio in the MCP varied with the Cit concentration in the medium or that the Ca fraction not integrated to the MCP was a function of pH, as proposed by Dalgleish and Law (1989), depending also on the Cit concentration.

In any case, the variation in the slopes of the lines obtained at different Cit concentration could not be directly associated with the stoichometric relation of Ca and P_i in the MCP. At pH low enough to completely dissociate the P_i, a fraction of Ca remained even associated with the micellar proteins, as shown by the intercepts of the lines in Figure 3. This fraction increased at increasing Cit concentration, coincidently with the increase of the Cit incorporated to the micellar structure at the same pH. This fact could be indicating the formation of Ca−Cit complexes able to be bound to casein residues.

CONCLUSIONS

In summary, the study of the incorporation of different mineral and proteins to ACM formed at different Cit concentrations produced the following observations:

At constant concentrations of caseins, Ca, and P_i in the medium, their incorporation to ACM was conditioned by the Cit concentration, probably as a result of the regulation of Ca activity by the chelating action of Cit. In the experimental conditions used, there was a

Cit concentration range in which the ACM size remained almost constant at its higher value.

The molar ratio at which Ca and P_i were incorporated to the ACM varied for different Cit concentrations. At decreasing pH, the Ca/P_i molar ratios for the remanent ions were dependent on the Cit concentration. These observations could be related to a certain kind of competition between Cit and groups able to bind Ca in the ACM, whose binding constants for this cation depend on the pH of the medium.

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