Properties of Dephosphorylated α_{s1}-Casein. Precipitation by Calcium Ions and Micelle Formation[†]

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ABSTRACT: The phosphate groups of α_{s1}-casein were removed by using phosphoprotein phosphatase from beef spleen. Dephosphorylated α_{s1}-casein like native α_{s1}-casein is precipitated by $CaCl_2$. For α_{s1}-casein a minimum number of calcium ions must be bound to initiate precipitation, the amount of calcium bound being a function of α_{s1}-casein concentration. With dephosphorylated α_{s1}-casein, however, precipitation by calcium is less dependent on the protein concentration. This suggests that less calcium is bound in the precipitation of the dephos-

phorylated α_{s1}-casein molecules. In the presence of Ca^{2+} and κ-casein, both α_{s1}- and dephosphorylated α_{s1}-caseins form colloidal complexes (casein micelles). However, examination of the micelles by electron microscopy reveals that the micelles formed from dephosphorylated α_{s1}-casein are three times the size of native α_{s1}-micelles and fewer in number. These observations suggest that calcium binding sites other than phosphate are involved in the formation of dephosphorylated α_{s1}-casein micelles.

α_{s1}-Casein, the major protein of cow's milk, contains eight phosphate residues and has a molecular weight of 23,600 (Grosclaude *et al.*, 1970; Mercier *et al.*, 1971). The phosphate groups occur as serine monoesters and the effect of these groups on the properties of α_{s1}-casein has been recently summarized by Waugh (1971). The α_{s1}-casein monomer has been assigned a net charge of -22 at pH 6.6, so that over one-third of this charge can be attributed to the phosphate groups (Ho and Waugh, 1965; Waugh *et al.*, 1971).

One of the characteristic features of α_{s1}-casein is its insolubility in the presence of calcium or other divalent cations. Polymer cross-linking of the phosphate groups with calcium is proposed as a likely mechanism for this precipitation (Waugh *et al.*, 1971). In the presence of κ-casein, α_{s1}-casein no longer precipitates on the addition of calcium ions but forms a stable colloid, known as the casein micelle (Waugh and von Hipple, 1956). The exact mechanism, by which up to 50,000 casein monomers are assembled into this protein complex, is unknown. Ionic bonds between the phosphate groups of the casein and calcium ions have been implicated in all the proposed models for casein micelle formation (Rose, 1969).

Limited research has been done on dephosphorylated casein. Pepper and Thompson (1963) first demonstrated that the removal of phosphate groups from α_{s1}-casein decreased its ability to form a stable complex with κ-casein. Recently, Dickson and Perkins (1971) showed that less calcium is bound to dephosphorylated α_{s1}-casein than to native α_{s1}-casein.

Our objective has been to prepare dephosphorylated α_{s1}-casein and to compare its properties to native α_{s1}-casein in two respects: (1) precipitation by calcium ions, and (2) casein micelle formation.

Experimental Section

Materials. Deionized water, prepared by the passage of distilled water over a mixed-bed cation–anion exchange resin, was used throughout the study. α_{s1}-Casein B, as defined by

Thompson *et al.* (1965), was isolated from the milk of an individual cow. Sephadex G-75[1] was purchased from Pharmacia Fine Chemicals, Inc.

Electron Microscopy. Micelles were prepared for electron microscopy by the method of Carroll *et al.* (1968) using 1% glutaraldehyde as the fixative. Micrographs were taken with an RCA EMU 3-G microscope using a 50-μ aperture at 100-kV acceleration voltage.

Zonal Electrophoresis. Electrophoretic patterns were obtained using 7% polyacrylamide gel in 4.5 M urea with Tris buffer (pH 9.2) applying a maximum voltage of 350 V at 80 mA (Thompson *et al.*, 1964). β-Mercaptoethanol was added to all samples before electrophoresis.

High-Voltage Paper Electrophoresis. Electrophoresis was carried out on Whatman No. 3MM paper for 3 hr at 3.5 kV, using a pyridine–acetate buffer at pH 6.4 (Kalan *et al.*, 1966).

Preparation of Proteins. Phosphoprotein phosphatase was prepared from beef spleen by the method of Revel and Racker (1960). The preparation was kept frozen in 5 mM sodium chloride–2 mM acetate solution (pH 6.0). Crude α_{s1}-casein was prepared by the method of Aschaffenburg (1963) and was purified on DEAE-cellulose (Thompson, 1966). The α_{s1}-casein B showed a single band on electrophoresis. κ-Casein was prepared by the method of Craven (1970) from pooled milk.

Solubilities of Caseins. The solubility of α_{s1}-casein in $CaCl_2$ was determined at 37° according to the procedure of Thompson *et al.* (1969). Casein was dissolved in 20 mM imidazole-HCl buffer (pH 7.0) and adjusted to pH 7 with 0.1 N NaOH. The casein was dialyzed overnight against the imidazole buffer and brought to a final concentration of 20 mg/ml. Calcium chloride solutions at various concentrations were prepared in 0.14 M KCl. The protein and calcium chloride solutions were brought to 37° and mixed (0.2 ml of $CaCl_2$ and 0.2 ml of protein). The solution was then incubated in a 37° water bath for 15 min and centrifuged for 15 min at 13,000g at 37° in the Model L4 Beckman ultracentrifuge using an SW-56 rotor. Protein was determined on a 0.1-ml aliquot of the supernatant.

Stabilization of α_{s1}-Casein. Stabilization of α_{s1}-casein by

[1] Mention of products or companies does not constitute an endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

κ-casein in the presence of Ca^{2+} (0–0.02 M) was performed by the method of Noble and Waugh (1965). All solutions were prepared in 10 mM imidazole-HCl buffer (pH 7.0) (with or without 0.07 M KCl). Solutions containing 80 mg of α_{s1}-casein were prepared with and without κ-casein (5.0 mg) in a final volume of 4 ml. Aliquots of these protein solutions were brought to 37°, mixed with an equal volume of $CaCl_2$ and incubated at 37° for 15 min. The calcium caseinate solutions were then centrifuged at room temperature for 2 min at 480g in a Sorvall RC-2 centrifuge using an SS-34 rotor. At this centrifugal force, the colloidal calcium caseinate was not sedimented. Protein concentration of the supernatant was determined. For the α_{s1}–κ-casein mixtures, the initial κ-casein concentration was subtracted from the concentration of the supernatant protein.

Simulated Micelles. Micelles were prepared, as described above, in detail (see Stabilization of α_{s1}-Casein). These simulated micelles contained 1% α_{s1}-casein (native or dephosphorylated), 0.125% κ-casein, 0.01 M $CaCl_2$, and 0.01 M imidazole-HCl buffer, (without KCl), pH 7.0 at 37°. The solution was centrifuged for 2 min at 480g and the supernatant was retained for electron microscopy.

Protein Concentration. Protein concentration was determined in the presence of 0.1 M sodium citrate. The citrate served to reduce the turbidity of the calcium casein solutions. Samples were read at 280 nm in a Beckman DU spectrophotometer. The extinction coefficient at 280 nm was considered to be 1.0 cm^2/mg for both α_{s1}-casein and κ-casein (Noble and Waugh, 1965). It was assumed that the extinction coefficient of dephosphorylated α_{s1}-casein was identical to α_{s1}-casein.

Phosphorus Determinations. Inorganic phosphorus was measured by the procedure of Martin and Doty (1949). Organic phosphorus was determined by the method of Meun and Smith (1968).

Isoionic Point. Casein was deionized by stirring 2 hr with mixed bed resin (Amberlite MB-1 and Amberlite IR-120, 20:1) and the pH of the colloidal solutions was determined (Ho and Waugh, 1965).

DEPHOSPHORYLATION OF α_{s1}-CASEIN

Some of the parameters affecting the dephosphorylation of α_{s1}-casein, as well as the specific conditions used for the experiments, are described in Table I. Substrate concentration is the critical factor in achieving total dephosphorylation. At high casein concentration (1.55%), complete dephosphorylation is unobtainable in a finite period of time; however, the reaction goes easily when the casein concentration is 0.155%.

The following procedure was used for preparing large amounts of dephosphorylated α_{s1}-casein. α_{s1}-Casein (310 mg) was dissolved in water, adjusted to pH 6 with 0.1 N HCl, and diluted to a final volume of 100 ml. Then 0.5 ml of 2-mercaptoethanol and 1 ml of phosphoprotein phosphatase stock solution were added. The reaction mixture was incubated at 37° for 90 min to obtain partially dephosphorylated casein and for 5 hr to obtain totally dephosphorylated casein. At the end of the specified time, the solution was cooled to 4°, adjusted to pH 8.0 to stop the reaction, and lyophilized. At this stage, the preparation was monitored by gel electrophoresis to ascertain whether the dephosphorylation was complete; if it was not, the reaction was continued.

Proteolytic digestion was checked by high-voltage electrophoresis using a 2.0-mg aliquot of the undialyzed, lyophilized preparation. No evidence of small peptides was obtained.

Gel filtration was used to remove the inorganic phosphate

TABLE I: Factors Affecting the Dephosphorylation of α_{s1}-Casein.[a]

Casein (mg)	Enzyme (μl)	Time (min)	PO_4 Liberated (μg)	PO_4 Liberated (%)
1.55	10	60	15.4	100
3.1	10	60	15.9	51
15.5	10	30	10.0	6.5
15.5	10	60	19.0	12.3
15.5	50	30	21.7	14.0
15.5	50	60	27.2	17.5

[a] Test system: α_{s1}-casein adjusted to pH 6.0, 100 μmoles of cacodylate buffer (pH 6.0), 16 μmoles of 2-mercaptoethanol, and phosphoprotein phosphatase in a final volume of 1 ml incubated at 37°.

from the dephosphorylated α_{s1}-casein. The lyophilized protein was dissolved in 25 ml of 0.1 M ammonium bicarbonate (pH 8.3). The solution was passed through a Sephadex G-75 column (2.5 × 75 cm), equilibrated with the same buffer. The α_{s1}-casein was well separated from the inorganic phosphate under these conditions. The α_{s1}-casein fraction was dialyzed for 18 hr against distilled water and lyophilized.

Results

Characterization of Native and Dephosphorylated α_{s1}-Casein. The electrophoretic mobilities of the various preparations of α_{s1}-casein are shown in Figure 1. Native α_{s1}-casein is represented as a single band with the fastest electrophoretic mobility. Five bands can be seen in the partially dephosphorylated casein, while the totally dephosphorylated α_{s1}-casein is, to a large extent, a single band with the slowest mobility. α_{s1}-Casein has eight phosphate residues per mole (Mercier et al., 1971). The multiple bands seen in the partially dephosphorylated α_{s1}-casein probably represent α_{s1}-casein with varying amounts of phosphorus. The removal of phosphate groups should increase the isoelectric point of the α_{s1}-casein by decreasing its net negative charge. Thus, as phosphate groups are removed from the α_{s1}-casein, its electrophoretic mobility in the alkaline gel is correspondingly decreased.

Phorphorus analyses of the caseins shown in the electrophoresis pattern are 1.04, 0.3, and 0.05% for native α_{s1}-casein, partially dephosphorylated α_{s1}-casein, and totally dephosphorylated α_{s1}-casein, respectively. Thus, 95% of the phosphorus was removed from the native α_{s1}-casein.

The isoionic point determined for α_{s1}-casein B is 4.92, which is close to the published value of 5.05 (Thompson et al., 1969), whereas the isoionic point of the dephosphorylated α_{s1}-casein B is 5.88. Thus, the removal of phosphate groups from α_{s1}-casein increases the isoionic point by approximately 1 pH unit.

Solubility of α_{s1}-Casein in $CaCl_2$ Solutions. The solubilities of α_{s1}-casein and dephosphorylated α_{s1}-casein as a function of Ca^{2+} concentration are compared in Figure 2. Two concentrations of casein were used for the study—10.6 and 5.3 mg per ml (0.45 and 0.225 mM, respectively). Both native and dephosphorylated α_{s1}-casein are precipitated by calcium. Considering only native α_{s1}-casein, calcium is first added to α_{s1}-
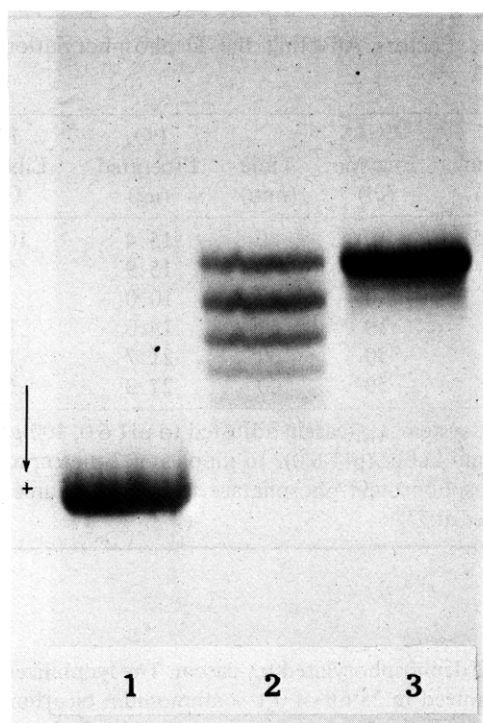
FIGURE 1: Polyacrylamide gel electrophoresis of (1) native α_{s1}-casein, (2) partially dephosphorylated α_{s1}-casein, and (3) totally dephosphorylated α_{s1}-casein. A Tris–urea buffer system (pH 9.2) with 7% polyacrylamide gel was used; the gel was stained with Amido Black (Thompson *et al.*, 1964).
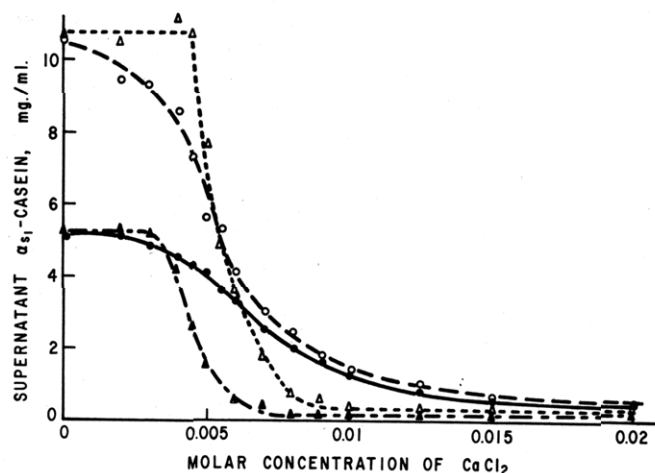

FIGURE 2: Solubility of calcium α_{s1}-caseinate at 37° as a function of increasing $CaCl_2$ concentration in the presence of 0.07 M KCl. The solutions contained 0.01 M imidazole-HCl buffer (pH 7.0). Curves represent native α_{s1}-casein and dephosphorylated α_{s1}-casein, using two different protein concentration. ($\triangle$) α_{s1}-Casein (10.6 mg/ml); ($\blacktriangle$) α_{s1}-casein (5.3 mg/ml); ($\bigcirc$) dephosphorylated α_{s1}-casein (10.6 mg/ml); ($\bullet$) dephosphorylated α_{s1}-casein (5.3 mg/ml).

casein to form a soluble calcium caseinate; then additional calcium produces an insoluble calcium caseinate. Thus, casein occurs in three forms: soluble casein, soluble calcium caseinate, and insoluble calcium caseinate.

The formation of soluble calcium caseinate can be observed in Figure 2; no precipitation of native α_{s1}-casein at either concentration occurs at low calcium ion concentrations. The point of precipitation of the α_{s1}-casein is displaced along the abscissa with increasing protein concentration and this displacement is related to the amount of soluble calcium caseinate formed. This displacement is much more evident in the native α_{s1}-casein curves, which show that more $CaCl_2$ is required to initiate precipitation using 10.6 mg/ml of casein than 5.3 mg/ml. The second displacement, which occurs after the casein has precipitated, represents the total calcium bound to the precipitated protein. Using a similar approach, Noble and Waugh (1965) pointed out that if there were no calcium binding to α_{s1}-casein, the solubility curves would be independent of protein concentration. By this criteria, dephosphorylated α_{s1}-casein shows much less evidence of calcium binding.

The graph in Figure 2 can be used to estimate the calcium binding of native α_{s1}-casein. Assuming a molecular weight of 23,600, the first displacement between the high and low concentrations of α_{s1}-casein indicates that 7 moles of calcium are bound per mole of protein prior to precipitation, while the binding of an additional 3 moles of calcium results in precipitation. These values agree with the results of Noble and Waugh (1965), who report 6–8 moles of calcium for the first displacement and 2–5 moles for the second displacement with an average value of 11 calcium ions bound per molecule. Dickson and Perkins (1971) obtained similar results using the radio-isotope (^{47}Ca). They estimated that native α_{s1}-casein binds

8.5 moles of calcium, while dephosphorylated α_{s1}-casein binds 1.2 moles of calcium.

Although there is no evidence of calcium binding prior to precipitation in the dephosphorylated α_{s1}-casein, the second displacement shows that 2 moles of calcium are bound per mole of dephosphorylated α_{s1}-casein. As 95% of the phosphorus has been removed from the α_{s1}-casein, any calcium must bind to anionic groups other than phosphate, such as the carboxylate groups of glutamic and aspartic acid. The α_{s1}-casein shows 11 binding sites, although Mercier *et al.* (1971) have reported only 8 phosphate residues/mole, indicating that α_{s1}-casein has calcium binding sites other than the phosphate residues.

Solubility of κ–α_{s1}-Caseins in $CaCl_2$ Solutions. A characteristic feature of α_{s1}-casein is that while it is insoluble in the presence of Ca^{2+}, κ-casein can prevent its precipitation by forming a stable colloid. Our finding that dephosphorylated α_{s1}-casein is also insoluble in the presence of Ca^{2+} prompted us to determine whether dephosphorylated α_{s1}-casein would also form a colloidal complex in the presence of κ-casein and Ca^{2+}. The effect of κ-casein on the solubility of α_{s1}-casein as a function of calcium ion concentration in the presence of 0.07 M KCl can be seen in Figure 3. When α_{s1}-casein and κ-casein are combined at a ratio of 8:1, the formation of a soluble complex is observed for the native α_{s1}-casein, but not for the dephosphorylated α_{s1}-casein. The dip which occurs in the native α_{s1}–κ-casein curve represents (as proposed by Noble and Waugh) α_{s1} which has not interacted with κ-casein on the descending limb and α_{s1} complexed with κ-casein on the ascending limb. The pronounced dip in the α_{s1}–κ-casein curve is due to the experimental conditions used, as KCl has been shown to enhance the dip (Thompson *et al.*, 1969; Noble and Waugh, 1965). Micelle formation occurs on the ascending limb of the dip and is very evident from the milky appearance of the native α_{s1}–κ-casein solutions when the $CaCl_2$ concentration exceeds 0.009 M. Although some increase in the solubility of dephosphorylated α_{s1}-casein is evident when κ-casein is present, the milky appearance accompanying micelle formation was not observed indicating that the removal of the phosphate groups has impaired the formation of casein micelles.
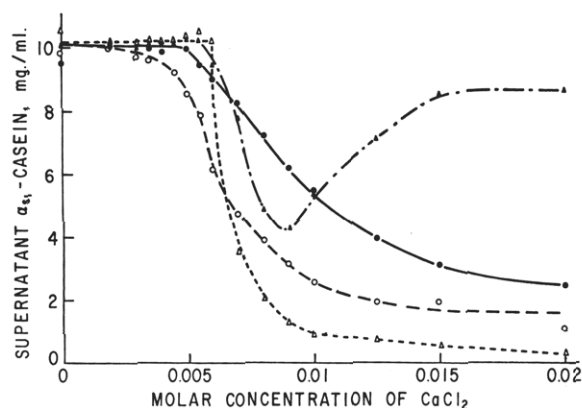
FIGURE 3: Effect of κ-casein on the solubility of α_{s1}-casein at 37° as a function of increasing CaCl$_2$ concentration in the presence of 0.07 M KCl. The solutions contained 0.01 M imidazole-HCl buffer (pH 7.0) and, initially, 10 mg/ml of α_{s1}-casein (native or dephosphorylated) with or without κ-casein (1.25 mg/ml). (▲) α_{s1}-κ-Casein; (●) dephosphorylated α_{s1}-κ-casein; (△) α_{s1}-casein, no κ-casein; (○) dephosphorylated α_{s1}-casein, no κ-casein.
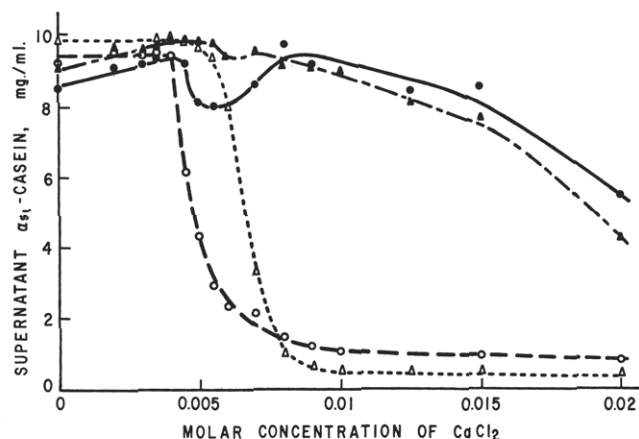

FIGURE 4: Effect of κ-casein on the solubility of α_{s1}-casein at 37° as a function of increasing CaCl$_2$ concentration in the absence of KCl. The solutions contained 0.01 M imidazole-HCl buffer (pH 7.0) and, initially, 10 mg/ml of α_{s1}-casein (native or dephosphorylated) with or without κ-casein (1.25 mg/ml). (▲) α_{s1}-κ-Casein; (●) dephosphorylated α_{s1}-κ-casein; (△) α_{s1}-casein, no κ-casein; (○) dephosphorylated α_{s1}-casein, no κ-casein.

If KCl is omitted and the above experiment is repeated, both casein preparations (native α_{s1}- and dephosphorylated α_{s1}-) remain soluble in the presence of κ-casein as shown in Figure 4 although showing some decrease in solubility when the Ca^{2+} exceeds 0.01 M. On the other hand, both preparations precipitate when κ-casein is omitted. The precipitation curve of native α_{s1}-casein (no added κ-casein) does not seem to change when KCl is omitted. Conversely, dephosphorylated α_{s1}-casein precipitates at a lower CaCl$_2$ concentration and more abruptly when the KCl is omitted. The results indicate that the removal of phosphorus from α_{s1}-casein does not affect its ability to form a stable colloid in the presence of κ-casein and Ca^{2+} in the absence of KCl. In both cases colloidal formation was also evident from the appearance of the supernatant solutions. Both κ-α_{s1}-casein solutions (native and dephosphorylated) became milky in appearance when the Ca^{2+} exceeded 0.006 M. It is interesting that a dip is present in the dephosphorylated α_{s1}-κ-casein curve and occurs at a calcium concentration of 0.006 M. The absence of a pronounced dip in the native α_{s1}-κ-casein curve is due to the low ionic strength, as KCl was omitted from these experiments. The fact that dephosphorylated α_{s1}-casein is stabilized by κ-casein, that a dip occurs in the stabilization curve and that the stabilization is accompanied by a colloidal appearance suggests that the interactions of κ-α_{s1}-casein and Ca^{2+} may be similar in both casein preparations.

Electron Microscopy of Simulated Casein Micelles. To determine whether the Ca-α_{s1}-κ-casein colloids formed with the dephosphorylated α_{s1}-casein were similar to those formed with native α_{s1}-casein, simulated casein micelles were examined by electron microscopy. The supernatant solution of the α_{s1}-κ-casein complexes (8:1) formed in 0.01 M CaCl$_2$ was used as a source of micelles. In Figure 5A, the α_{s1}-κ-casein micelles have an average diameter $\sim$2200 Å (range 1810–2530), somewhat higher than micelles from skim milk, which are $\sim$1400 Å (Carroll *et al.*, 1968). The small particles in the background average $\sim$230 Å. When dephosphorylated α_{s1}-κ-casein micelles were formed (Figure 5B) there was a significant increase in micelle diameter (average $\sim$6630 Å; range 3490–7590 Å), but a significant decrease in the number of micelles formed. Many more small background particles were

observed with an average diameter of $\sim$120 Å, which is about one-half the diameter observed in Figure 5A.

The micelles formed were separated from the background material by centrifugation (37,000g, 1 hr, 26°). This separation was confirmed by electron microscopy; only background material (no micelles) was observed in the supernatant solution. Determination of supernatant protein indicated that 74% of the native protein existed as α_{s1}-κ-casein micelles. However, only 46% of the dephosphorylated α_{s1}-κ-protein formed micelles, leaving 54% as nonmicellar background material which was not sedimented under these conditions. Gel electrophoresis indicated that the precipitate and the supernatant solutions both contained κ-casein as well as α_{s1}-casein (native or dephosphorylated).

Discussion

Native α_{s1}-casein has a net charge of -22 at pH 6.6. When enough calcium is added to initiate precipitation, the net
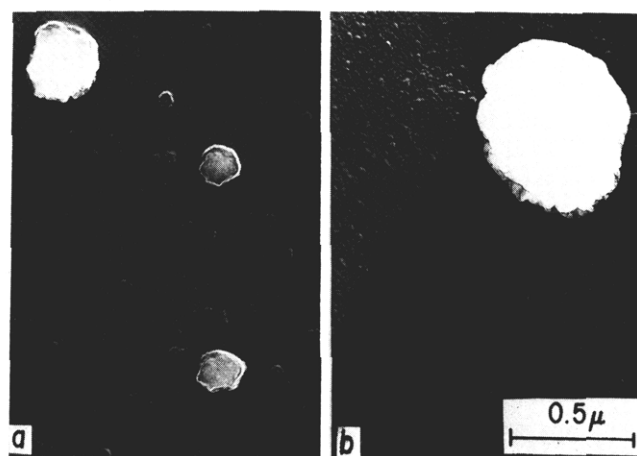

FIGURE 5: Electron micrograph of simulated milk micelles. Solutions contained 1% α_{s1}-casein (native or dephosphorylated), 0.125% κ-casein, 0.01 M CaCl$_2$, and 0.01 M imidazole buffer (pH 7.0). (A) α-$_{s1}$-Casein; (B) dephosphorylated α_{s1}-casein. The solutions were fixed in gluturaldehyde as described by Carroll *et al.* (1968).

charge is reduced to −9 (Waugh *et al.*, 1971). If eight phosphate groups are removed from α_{s1}-casein, the dephosphorylated α_{s1}-casein must have a charge of −10 to −14, depending on the ionization of the second hydroxyl group of the monoester phosphate groups. If charge reduction is the essential factor in the precipitation of dephosphorylated α_{s1}-casein, then its precipitation should occur at a lower calcium ion concentration, as there are fewer negative charges to neutralize. However, this does not seem to occur (Figure 2). If the calcium chloride in Figure 2 is replaced by KCl (ionic strength, 0.1–0.35 μ), no precipitation occurs with either native or dephosphorylated α_{s1}-casein. Therefore, it seems likely that calcium binding occurs in the dephosphorylated α_{s1}-casein and forms bridges between adjacent polymers binding negative groups other than phosphates. As a result, precipitation occurs. Dickson and Perkins (1971) have shown that dephosphorylated α_{s1}-casein binds one to two calcium ions per mole while κ-casein binds up to 3 moles of calcium. Dephosphorylated α_{s1}-casein has a higher net charge and binds fewer calcium ions than κ-casein, yet the dephosphorylated α_{s1}-casein precipitates, whereas κ-casein does not. Therefore, the precipitation cannot be a simple charge reduction by divalent ions.

The calcium precipitation of native α_{s1}-casein is relatively insensitive to ionic strength as seen by comparing Figures 3 and 4. On the other hand, the precipitation of dephosphorylated α_{s1}-casein is sensitive to added KCl, as shown by the fact that more calcium is required to precipitate dephosphorylated α_{s1}-casein when KCl is present. This suggests that calcium binding forces are weaker in the dephosphorylated α_{s1}-casein. For the same reason, the forces that are responsible for micelle formation are weaker in the dephosphorylated casein, as added KCl markedly reduces the solubility of dephosphorylated casein in the presence of κ-casein.

The results shown in Figure 4 indicate that both native and dephosphorylated α_{s1}-caseins are stabilized against calcium precipitation by κ-casein in the absence of KCl. However, Pepper and Thompson (1963) concluded that dephosphorylation of α_{s1}-casein drastically impairs its ability to be stabilized by κ-casein in 0.02 M $CaCl_2$ at pH 7.0. (No buffer and no KCl were added.) Although our results seem to contradict their conclusions, the two views are not incompatible. They varied the κ–α_{s1} ratio from 0 to 0.12 keeping the Ca^{2+} constant (0.02 M). At low concentrations κ-casein did not stabilize dephosphorylated α_{s1}-casein as effectively as native α_{s1}-casein. However, if one reexamines their curve and extrapolates to slightly higher κ–α_{s1} ratios, complete stabilization of dephosphorylated α_{s1}-casein would probably occur. In the results presented here, the κ–α_{s1} ratio is 0.125 and the Ca^{2+} varies from 0 to 0.02 M.

The stabilization of α_{s1}-casein by κ-casein in the presence of Ca^{2+} is usually explained as the formation of a soluble complex known as the casein micelle. Such complexes are formed (see Figure 5) by both the native and the dephosphorylated α_{s1}-caseins. Up to 74% of the native α_{s1}-casein is incorporated into micelles. However, only 46% of the dephosphorylated α_{s1}-casein is incorporated into micelles. Fifty-four per cent of

the protein occurs as small particles (background particles observed by electron microscopy). Calcium binds to phosphate groups more strongly than to other groups (such as aspartic and glutamic residues). Hence the lack of phosphate binding sites on dephosphorylated α_{s1}-casein would weaken the forces necessary for complete micelle formation, thus causing fewer micelles, as well as stabile, nonsedimented, background particles (Figure 5B).

Although our results indicate that micelle formation is reduced when the phosphorus is removed from α_{s1}-casein, it is significant that micelles are still formed. A reasonable explanation would be that calcium binding sites other than phosphate are important in forming interpolymer calcium bonds which gives the casein micelle structural stability.

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