Casein Micelles. Formation and Structure. II[1]

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Studies of the formation and structure of casein micelles have revealed experimental facts which any model must accommodate. These are: (A) Prior to micelle formation at 37° there are present free calcium α_s-caseinate and a low weight ratio calcium α_s–κ-caseinate interaction product. (B) For stability, micelles require a minimum level of calcium which is greater than that required to precipitate the free α_s-casein present. (C) The more centrifugable (larger) the micelle the lower its weight fractional content of κ-casein. (D) Precipitates formed in the presence of κ-casein and at calcium concentrations sufficient for micelle stability are different from calcium α_s-caseinate precipitates in being nonadherent and compacting to give white opaque pellets. These precipitates contain small amounts of κ-casein. Calcium α_s-caseinate precipitates treated with κ-casein become nonadherent. (E) Micelles are stable with respect to the close approach induced by sedimentation into a pellet. (F) In all cases micelles have a size distribution which depends strongly on the initial ratio, the calcium concentration on single aliquot addition, and the initial protein concentration. The apparent final states of the systems are dependent on the path. Path dependencies were found for assay supernatant protein, supernatant protein after ultracentrifugation, and a wide variety of micelle–precipitate combinations obtained either by single aliquot or incremental addition of calcium. (G) Complete stabilization in the region of the peak can be achieved at all α_s/κ initial weight ratios up to 10 and micelle populations stable with respect to assay conditions may have weight ratios in excess of 10. (H) The size distribution of micelles can be altered by the addition of κ-casein and probably by the addition of calcium. Size changes induced by κ-casein addition occur rapidly (minutes) while those attending calcium addition take hours. κ-Casein has some capacity to solubilize calcium α_s-caseinate precipitate but the resulting supernatant α_s/κ weight stabilization ratios are small (0.5–2). (I) Stabilizing capacity is available in some micelle systems after the systems have come to apparent final states. (J) A micelle distribution, once formed, is relatively stable to dilution with a buffer containing an appropriate calcium concentration. (K) Micelles are highly solvated and the solvation decreases as the initial ratio increases. (L) Although micelles require the presence of α_s- and κ-caseins, they can incorporate variable amounts of β-casein. With these facts in mind, models of the micelles as single phase particles or large chemical compounds are unattractive. The following model is proposed. Micelles consist, in simplest form, of cores of calcium α_s-caseinate covered by a uniform coat of low weight ratio calcium α_s–κ-caseinate. Calcium α_s-caseinate in the core need not be in contact, therefore in exchange, with the environment. The carbohydrate moiety of κ-casein is placed to the outside where little tendency to interaction is required. Internally the coat subunits have a strong interaction with core calcium α_s-caseinate and probably a lateral preference for each other. Surface components are in exchange with similar components in solution. Kinetically, random processes lead to the production of calcium α_s-caseinate particles and their acquisition of a coat. The calcium dependency of coat formation is greater than that of calcium α_s-caseinate precipitation. There is little free calcium κ-caseinate or calcium α_s-caseinate in solution in contact with micelles. There may be variable amounts of excess coat material in the form of coreless micelles or other interaction products. The coat itself has a calcium instability and therefore a calcium dependency less than that of products having a lower weight ratio than the coat subunit. The final member of the latter series could be a form of pure calcium κ-caseinate. The solvation of micelles is reasonable from the solvation characteristics of components. The model accepts β-casein to an extent. It places the carbohydrate moiety of κ-casein to the outside where it is accessible, even in extraordinarily stable micelles, to the action of rennin.

Introduction

In part I of this series[3] we have referred to the pioneering work of Linderstrøm-Lang[4,5] and to the persistence of his fundamental postulate that a casein nonprecipitable by calcium (insensitive) stabilizes, in some way, calcium-precipitable casein.

There is now no doubt that κ-casein, a component amounting to approximately 15% of the total casein, can stabilize the remainder, most of which is remarkably insoluble in the presence of calcium ion at 37°. The necessary and sufficient conditions for micelle formation, however, are α_s-casein, κ-casein, and a divalent cation.[6] In part I of this series we have shown that in the absence of divalent cation a solution containing α_s- and κ-caseins exists at low temperatures as a mixture of α_s-casein polymers and κ-casein polymers with little or no evidence of interaction, even at a low weight ratio of interactants. At 37° a variety of interactions takes place but the only interactant that can possibly survive prior to the formation of micelles is one having a weight ratio near unity. The remainder of the α_s-casein is free to precipitate as calcium α_s-caseinate. These data, paralleled by data obtained on First Cycle casein and Solubilized Skim Milk, negate the existence of a preformed α_s–κ-complex whose final stoichiometry limits the maximum stabilization of α_s-casein.

(1) This work was supported by Grant No. GM 05410 from the Division of Research Grants, National Institutes of Health.

(2) Predoctoral Fellow of the National Science Foundation and Postdoctoral Fellow of the National Institutes of Health. A portion of this work was submitted in partial fulfillment of the requirements for the Ph.D. degree at the Massachusetts Institute of Technology in 1964.

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At the time the existence of a preformed complex was being re-examined, data were also being accumulated which suggested that an initial weight ratio of 4 (α_s/κ) was representative of a range of initial ratios which gave particularly stable micelles. More recently Zittle, *et al.*,[7] Noble,[8] and Noble and Waugh[9] have found stabilization of α_s-casein by κ-casein at weight ratios far in excess of 4. Indeed, complete stabilization at 0.02 *M* calcium can be obtained at an initial ratio of 10 and there is reason to believe that this may represent, in its turn, only the maximum ratio where the largest micelles are not centrifuged out under the conditions of assay.

One of our more important previous conclusions is that calcium drives not only the precipitation of calcium α_s-caseinate but also reactions involving α_s- and κ-caseins, the end product of a set of the latter interactions being a stable micelle.

Little is known about the details of micelle formation or structure. Micelles appear to be spherical[8,10,11]; they contain α_s-, β-, and κ-caseins as major components, and certaintly a variety of minor components in small amounts.[12,13] Micelles are obviously highly solvated,[14] have a size distribution ranging at least from 700 to 2800 Å. in diameter,[10] have a wide latitude for stability,[8] and have no obvious internal structure, as is shown by X-ray diffraction patterns obtained from micelle pellet centrifugates.[15]

Our purpose here is to present studies of mixtures of pure components and of Solubilized Skim Milk and First Cycle casein. These studies have led to a general model for mechanism of formation, structure, and stability of micelles. The model accounts also for important aspects of rennin coagulation.

Materials and Methods

The following materials and methods have been described by Noble and Waugh in Part I of this series[3]: skim milk, Solubilized Skim Milk, First Cycle casein, κ-casein, protein concentration, starch gel electrophoresis, and ultracentrifugation. Other materials and methods, including chemicals, laboratory distilled water, treatment of dialysis tubing, preparative ultracentrifugation, and $\alpha_{s1,2}$-casein (α_s-casein), have been described by Waugh and co-workers.[13]

Turbidity. Samples are examined in a Beckman D.U. spectrophotometer set at λ 3200 Å. Optical densities are corrected, when necessary, by determination of solvent readings. When readings exceed 1.0 O.D. unit, lower values are obtained by using quartz cell inserts which are then taken into account by an appropriate factor. Turbidity, τ_n, is expressed here as the observed optical density in a cell having a 1-cm. path length divided by the total protein concentration in mg./ml.

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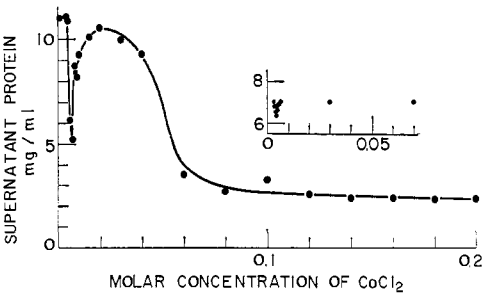

Figure 1. Supernatant protein resulting from single aliquot addition of calcium plotted as a function of $CaCl_2$ concentration for two α_s–κ-casein mixtures in standard KCl buffer at 37°. The main curve represents data for a solution initially containing 10 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein. The inset represents data for a solution initially containing 5 mg./ml. of α_s-casein and 2 mg./ml. of κ-casein. The designations of the segments of the curves in order of increasing $CaCl_2$ concentration are the dip, the peak, and the pseudo-plateau.

A change in turbidity could be due to alterations in average particle protein content (size), solvation, or shape. Significant changes in shape are not expected (see below). There is some reason to believe that changes in solvation may affect turbidity as noted under Micelle Solvation. Here an increased turbidity is associated with decreased solvation. When large decreases in sedimentation and turbidity occur it is likely that there has been a decrease in size.

Standard KCl Buffer. This solution contains 0.07 *M* KCl, 0.01 *M* imidazole and is at pH 7.1.

Assay for Supernatant Protein. This assay has been described in detail in part I of this series.[3] Note that the initial α_s-casein concentration divided by initial κ-casein concentration, both in mg./ml., is the *initial ratio.* The supernatant protein concentration minus the combination of the solubility of calcium α_s-caseinate and the initial κ-casein concentration is referred to throughout this work as the *degree of stabilization.* The degree of stabilization divided by the initial κ-casein concentration is referred to as the *stabilization ratio.* These last two values are accurate only if the precipitates contain no κ-casein (see below).

Assay for κ-Casein in the Presence of α_s-Casein. If the initial α_s-casein concentration is held constant at 10 mg./ml. and κ-casein is varied from 0 to 1.0 mg./ml., there occurs a large change in supernatant protein concentration using single aliquot addition of calcium to give 0.015 *M*. Figure 7 plots supernatant protein in mg./ml. of original solution vs. κ-casein concentration under these conditions. If, at 10 mg./ml. an α_s-casein solution originally contains some κ-casein, the curve will, of course, be shifted to the left on the abscissa by a distance nearly equal to the original κ-casein content; nearly equal because the α_s-casein concentration will not be quite 10 mg./ml. If the unknown α_s-casein contains 4% or less of κ-casein this effect constitutes a small perturbation.

The assay was applied to the determination of the κ-casein content of peak and plateau precipitates as follows: A tube containing precipitate was carefully drained, since the supernatant in some instances contained large amounts of κ-casein, and the sides of the tube were wiped with a lint-free cellulose sheet (Kimwipes). The precipitate was then dissolved in 0.05 *M* sodium citrate and dialyzed against 0.07 *M* sodium

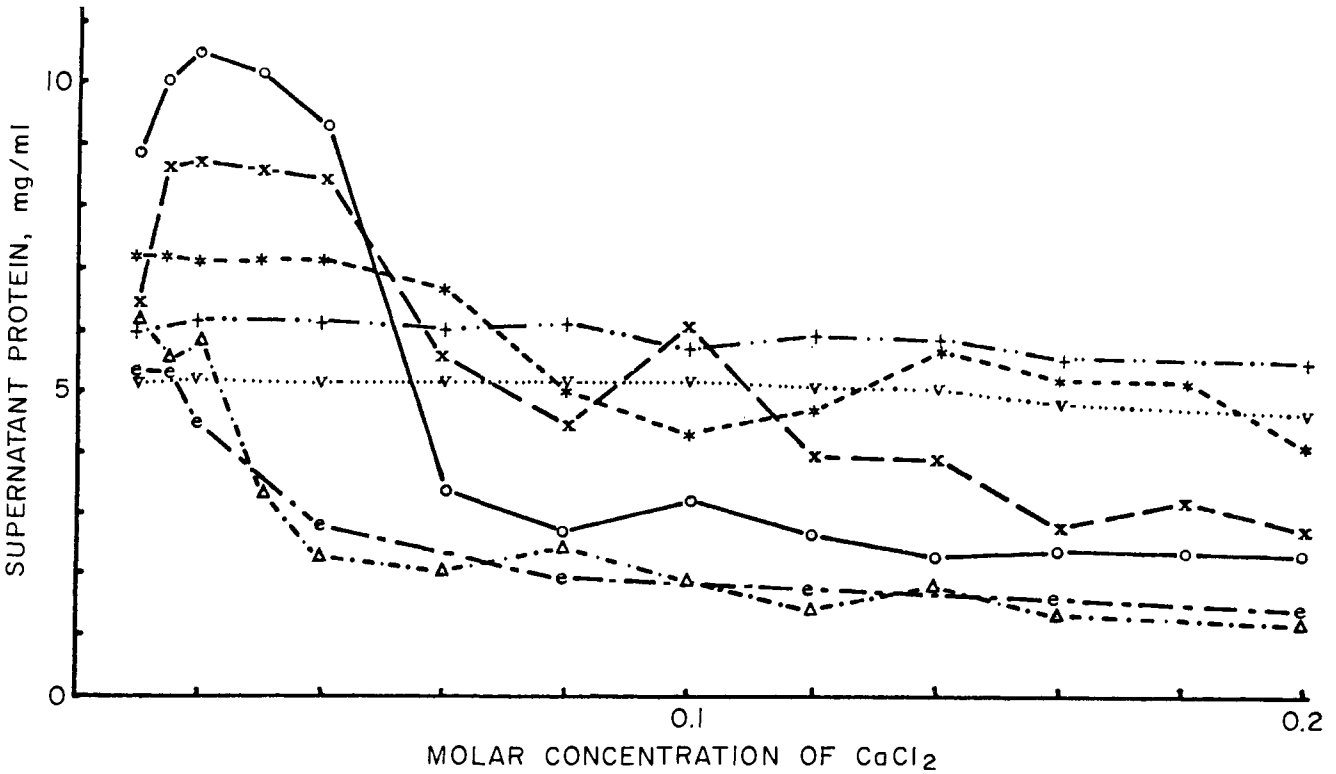

Figure 2. Supernatant protein resulting from single aliquot addition of calcium plotted as a function of $CaCl_2$ concentration for a series of α_s–κ-casein mixtures in standard KCl buffer at 37°. Each solution contained initially 1 mg./ml. of alcohol-fractionated κ-casein. The different initial α_s-casein concentrations are represented as follows: Δ for 20 mg./ml.; e for 15 mg./ml.; O for 10 mg./ml.; × for 8 mg./ml.; × for 6 mg./ml.; + for 5 mg./ml.; and V for 4 mg./ml.

chloride so that the final protein concentration was above 10 mg./ml. These solutions were mixed with 0.07 *M* sodium chloride and κ-casein in 0.07 *M* sodium chloride to fulfill the initial conditions of the assay.

Results

Illustrative Behavior of α_s–κ-Casein Mixtures upon Addition of Calcium. The main curve of Figure 1 shows supernatant protein as a function of total calcium concentration for a solution containing initially 10 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein in standard KCl buffer. At very low calcium concentrations (0 to 0.004 *M*) no precipitate forms and the turbidity of the solution does not visibly increase. From 0.004 to 0.006 *M* calcium there is a sharp decrease in supernatant protein, which at 0.006 *M* is about 5 mg./ml. Supernatants remain clear. From 0.006 to 0.015 or 0.02 *M* calcium, supernatant protein increases until no precipitate forms. From 0.02 *M* calcium upward supernatant protein decreases rapidly until 0.06 *M* calcium is reached, after which it decreases slowly. In the region of increasing supernatant protein between 0.006 and 0.015 *M* calcium a reproducible secondary dip may appear.

A standard nomenclature will be used to refer to the various regions of this curve. The region between 0.006 and 0.01 *M* calcium, where supernatant protein decreases sharply and then increases, will be known as the dip. The region between 0.007 and 0.04 *M* calcium, where supernatant protein goes through a maximum, will be referred to as the peak. The first region of decreasing supernatant protein is the descending limb of the dip; this is followed by the ascending limb of the peak, the top of the peak, the descending limb of the peak, and the pseudo-plateau.

Micelles are first apparent, as a visible increase in turbidity of the solution, near the bottom of the dip. Along with the increased supernatant protein which then follows there is an increase in turbidity until at approximately 0.01 *M* calcium the system has the appearance of skim milk.

Peak and Post-Peak Behavior on Single Aliquot Addition of Calcium. Effect of Initial Ratio. In Figure 2 is plotted supernatant protein *vs.* total calcium concentration for solutions containing 1 mg./ml. of κ-casein and varying concentrations of α_s-casein, leading to initial ratios between 20 and 4. Standard KCl buffer was used. The region of the dip, which has been considered in part I of this series,[3] has been omitted.

As the initial ratio is varied, while holding the κ-casein concentration constant, two changes are seen in the solubility behavior. First, in the region of the peak at 0.02 *M* calcium, 1 mg./ml. of κ-casein can stabilize completely 10 mg./ml. of α_s-casein. At an initial ratio of 8 the calcium range at the top of the peak over which little or no precipitate forms is from 0.015 to 0.04 *M;* for an initial ratio of 6 this range is from 0.01 to ~0.05 *M;* and for an initial ratio of 5 the range extends out to 0.07 *M* calcium. When the initial ratio is increased to 15 or 20, less stabilization is accomplished although there is still a small peak in both of these curves. For all points, subtracting the initial κ-casein concentration from supernatant protein leaves an amount that greatly exceeds the calcium α_s-caseinate solubility.[3] Second, in the region of the pseudo-plateau, stabilization at weight ratios of 5 or below is complete. As the initial ratio increases above 5 the supernatant protein pro-

gressively decreases leading, obviously, to decreases in the degree of stabilization and the stabilization ratio.

The individual supernatant protein curves do not appear to be monotonic. At this time the significance of these fluctuations is not known, but they appear to be due to experimental factors.

Effect of Initial Protein Concentration. In Figure 3 are shown plots of supernatant protein *vs.* calcium concentration (single aliquot addition) for solutions in which protein concentration was varied at constant initial ratio. The data in Figure 3a are for an initial ratio of 10, in Figure 3b for an initial ratio of 5, and in Figure 3c for First Cycle casein.

In all cases, in the region of the pseudo-plateau supernatant protein increases with increasing protein concentration. At the same time the per cent of initial protein remaining in the supernatant decreases. Thus, with increasing concentration the degree of stabilization increases but the stabilization ratio decreases.

As was found previously for studies of precipitation in the region of the dip,[3] First Cycle casein shows the same behavior as an α_s–κ-casein mixture having an initial ratio near 7.

Whatever the result of single aliquot addition, each system rapidly comes to a state which is stable, as far as precipitate formation is concerned, for as long as the experiment can be conducted. Experiments are eventually terminated after a few days as a result of enzyme or microbial action.

Stability of Final States. Effect of κ-Casein on Micelles and on Calcium α_s-Caseinate Precipitates. The addition of κ-casein to a micelle system produces a decrease in turbidity, most of which occurs within a few minutes. Differential ultracentrifugation reveals a concomitant decrease in pellet protein suggesting a decrease in micelle size. This was demonstrated as follows. A micelle system was established at 0.02 *M* calcium in standard KCl buffer. The system contained 10 mg./ml. of α_s-casein and 2 mg./ml. of κ-casein. This system was diluted to 3 mg./ml. of α_s-casein with standard KCl buffer containing 0.017 *M* calcium.[16] Aliquots were then taken and volumes of calcium κ-caseinate solution and standard KCl buffer, both 0.02 *M* in calcium, were added to give a final level of 2.4 mg./ml. of α_s-casein and total κ-casein concentrations of 0.48 (no added κ-casein), 0.96, 1.44, and 1.92 mg./ml. Turbidities were measured immediately and at 45-min. intervals. The sequence at 45 min. was τ_n = 2.2, 1.4, 0.90, and 0.63. At 45 min., 2-ml. samples were taken from each aliquot and centrifuged at 15,000 r.p.m. (SW 39 rotor) for 5 min. at 37°; then, after decanting into new tubes, the supernatants were centrifuged for 5 min. at 20,000 r.p.m. Two pellets were thus recovered and their protein contents were determined. The sums of these two pellets were 3.3, 3.9, 2.1, and 1.3 mg. in order of increasing κ-casein. For small added κ-casein, the first pellet showed the major change, while for large added κ-casein, both pellets decreased.

Calcium κ-caseinate has been added to the usual precipitate obtained by single aliquot addition to 0.02 *M* calcium, an initial ratio of 15, and 15 mg./ml. of α_s-casein. Experiments have been carried out in two ways: by adding calcium κ-caseinate either to the complete system or to the precipitate obtained after assay centrifugation. In each case 20 mg. of κ-casein in 0.57 ml. of standard KCl buffer containing 0.02 *M* calcium was added either to 2 ml. of the complete system or to the assay precipitate resulting from 2 ml. of this system. The assay precipitate contained 24.5 mg. of protein. After calcium κ-caseinate addition the systems were shaken in the standard way. Precipitates resuspended within a few minutes. After shaking for 5 hr. assay supernatant protein was determined. In both cases, assay precipitate had decreased by 60% and the final supernatants were calculated to have stabilization ratios of 0.9 and 0.8, respectively. Final assay supernatants visually had a low turbidity.

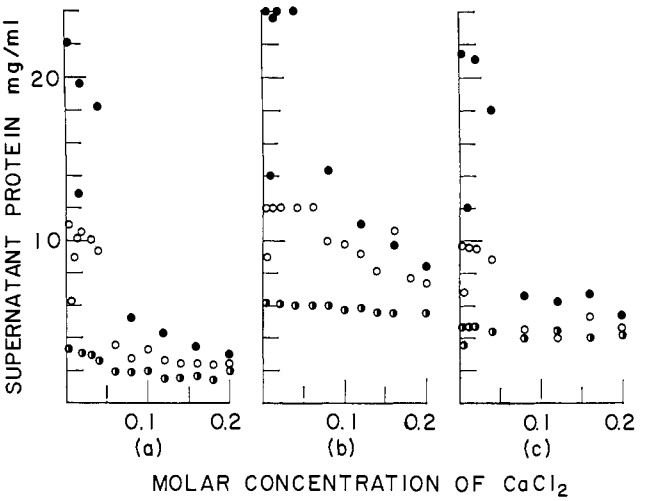

Figure 3. Supernant protein resulting from single aliquot addition of calcium plotted as a function of $CaCl_2$ concentration for initial ratios of 10 (a) and 5 (b) and for First Cycle casein (c) at several initial protein concentrations. The symbols are for initial concentrations where ● is for 20, ○ is for 10, and ◑ is for 5 in (b) and (c) and 3 in (a). The units are mg./ml. of α_s-casein for (a) and (b) and O.D. units/ml. for (c).

Attempts have been made to solubilize calcium α_s-caseinate precipitates by adding κ-casein to their supernatants. The result depends on the degree to which the calcium α_s-caseinate precipitate is consolidated prior to κ-casein addition and the extent to which it is sheared after κ-casein addition. Nonconsolidation coupled with gentle shearing can lead to apparent stabilization, as was found previously.[17]

The most critical test of solubilization was conducted as follows. Two α_s-casein aliquots were precipitated by calcium addition, after which κ-casein in solution was added. The systems finally had a volume of 1 ml., were in standard KCl buffer, 0.02 *M* in calcium, at pH 7, and contained 5 mg. of α_s-casein and added κ-casein to make the initial ratio either 1.0 or 1.5. Each system was shaken in the standard way. At both ratios solubilization occurred: at unit initial ratio to give stabilization ratios of 0.28 and 0.57 after 8 and 26 hr., respectively, and at 1.5 initial ratio to give stabilization ratios of 0.43 and 0.62. The calcium α_s-caseinate solubility was small compared to the α_s-casein solubilized. During shaking but before the addition of κ-casein the precipitates consolidated so that after 15

(16) This is the dialyzable calcium concentration using a total calcium concentration of 0.02 *M* for the system described. The calcium concentration is one of a series determined by Dr. L. K. Creamer using equilibrium dialysis and flame photometry as the method for determining calcium.

(17) D. F. Waugh, *J. Phys. Chem.*, 65, 1793 (1961).

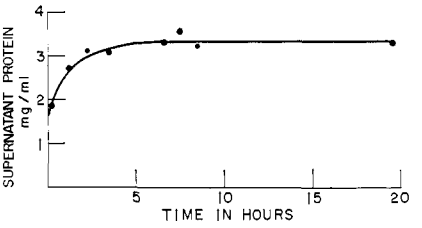

Figure 4. Solubilization of calcium α_s-caseinate by κ-casein: Supernatant protein plotted as a function of time when 10 mg./ml. of α_s-casein is precipitated by calcium addition and then 1 mg./ml. of κ-casein is introduced into the system. The final calcium concentration was 0.02 *M* (see text).

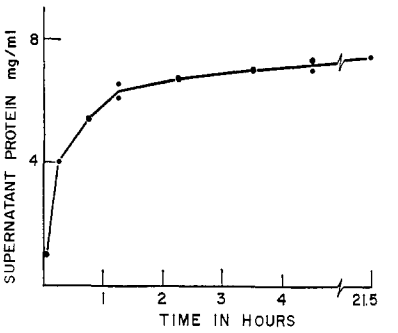

Figure 5. Resuspension of the pellet from a micelle system of initial ratio 10 centrifuged at 37° for 1 hr. at 30,000 r.p.m. (SW 39). Supernatant protein is plotted as a function of time. Complete resuspension corresponds to 11 mg./ml. of supernatant protein.

min. they were single transparent glass-adherent masses. After the addition of κ-casein these masses became nonadherent and slowly distorted into submasses joined by threads, some of which were very thin. Disruption of the precipitates, although slight, might contribute to the degree of stabilization as is evident from what follows.

A number of experiments were conducted on similar systems in water-jacketed beakers at 37°, stirred maximally but without foaming by a small Teflon-coated bar. In a typical experiment of final volume 10 ml., the system contained 100 mg. of α_s-casein in 9 ml. at 0.022 *M* calcium. One milliliter containing 10 mg. of κ-casein was then added. Just before κ-casein addition stirring produced compacted precipitate which adhered to the glass beaker and the stirring bar. After κ-casein addition the surface of the precipitate became nonadherent; in fact, larger masses broke away and were progressively fragmented. The appearance of the system approached that of milk as the initial precipitate disappeared. Most of the resulting particles were too large to remain in suspension during assay centrifugation. The presence of micelles, however, was indicated by assay supernatant turbidity. Assay supernatant protein is shown in Figure 4. The final stabilization ratio is ~2.5. Precipitates are expected to contain κ-casein and the true stabilization ratio is undoubtedly higher.

In similar experiments involving rapid stirring, carried out at initial ratios of 4 and 8, final stabilization ratios of 2.5 and 3.0 were obtained after 24 hr. Increasing the KCl concentration to 0.15 or 0.3 had no significant effect on final stabilization.

Previously[3] it was found that incremental addition of calcium to systems at initial ratios of 10 and 5 led to calcium α_s-caseinate precipitation at the start of the descending limb of the dip, as expected. However, this limb continued to decrease with further incremental addition to give final stabilization ratios of about 2. The latter did not change over a period of 15 hr.

Stability of Micelles to Close Approach. Close approach was accomplished by centrifugation in the swing bucket rotor, SW 39, at 37° for 30 min. at 30,000 r.p.m. Temperature was controlled accurately. After this treatment micelles were firmly packed into a pellet. The characteristics of portions of the pellets varied from soft gelatinous (top) to hard grainy (bottom), the amounts present depending on the nature of the system being centrifuged. Micelle integrity was tested by determining the extent to which a micelle suspension re-formed under conditions of gentle stirring or convection (to avoid diffusion as the rate-limiting step) when pellets were placed in contact with their corresponding supernatants.

A solution containing 10 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein (initial ratio 10) was made 0.02 *M* in calcium and centrifuged as described. The resulting rubbery pellet had a transparent upper surface and opaque base. Pellet and supernatant were placed in jacketed beakers at 37° and slowly stirred in such a way that supernatant flowed gently past the pellet without fragmenting it. Figure 5 records the supernatant protein, after the usual assay, *vs.* time. Within 1 hr. the supernatant protein increased from 1 mg./ml. to 7 mg./ml. After 24 hr. 8.5 mg./ml. were present out of a possible 11 mg./ml. Thus, at the maximum initial ratio for complete stabilization, 75% of the pellet had re-formed micelles within 24 hr.

At an initial ratio of 4, complete resuspension required less than 1 hr. At an initial ratio of 5, 90% of the pellet resuspended within 5 hr.

Carefully skimmed milk was centrifuged under the same conditions and pellet-to-micelle transformation was examined under conditions where the supernatant was either slowly stirred or held quiescent. Using slow stirring the pellet had essentially disappeared in 8 hr. When the pellet or the grainy lowest layer was suspended from a platinum hook in the quiescent supernatant, a micelle suspension streamed off the surface and accumulated, because of its increased density, in the bottom of the vessel. Similar results were obtained with a skim milk made 0.07 *M* in added calcium chloride.

If micelles are subjected to too high a centrifugal field, their capacity to resuspend is impaired. Micelle systems with initial ratios of 5 have been centrifuged in the SW 39 rotor for 30 min. at 35,000 r.p.m. The resulting pellets resuspended to the extent of about 50%.

The possible importance of the supernatant protein to resuspension was examined by comparing the resuspension of identical micelle pellets either in supernatant or in standard KCl buffer containing 0.017 *M* calcium. The pellets were obtained by centrifuging 5-ml. aliquots of a micelle system containing 5 mg./ml. of α_s-casein, 1 mg./ml. of κ-casein, 0.02 *M* calcium, and standard KCl buffer. After 5 hr. both pellets were 90% resuspended.

Sensitivity of Micelles to Added Calcium. A preformed micelle shows considerable stability on the further addition of calcium. Studies were carried out by

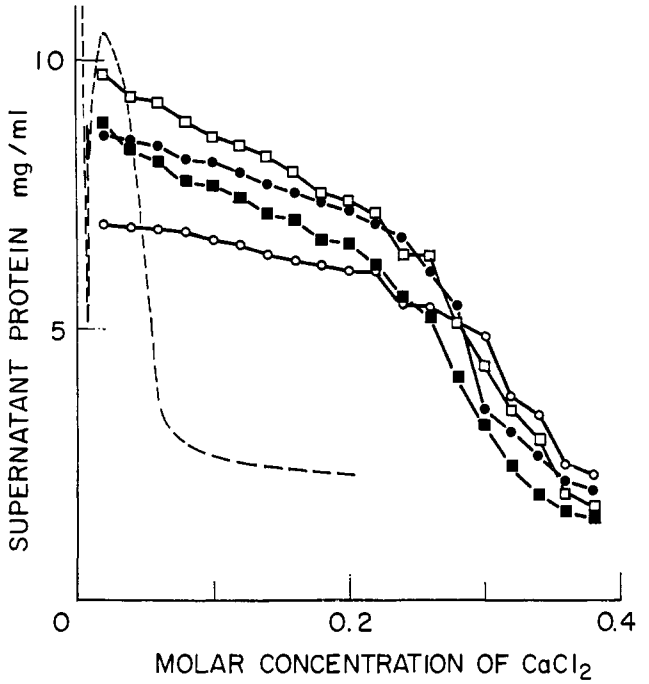

Figure 6. Results of incremental addition of calcium to micelle systems established at 0.02 *M* calcium when the initial protein concentrations are 1 mg./ml. of κ-casein and, respectively, 12 mg./ml. of α_s-casein, ■; 10 mg./ml. of α_s-casein, □; 8 mg./ml. of α_s-casein, ●; and 6 mg./ml. of α_s-casein, O. The single aliquot addition curve at an initial ratio of 10 is included for comparison (dotted line).

adding, first, single aliquots of calcium to give 0.02 *M* using α_s–κ-casein mixtures at 1 mg./ml. of κ- and α_s-casein to give different initial ratios. Using the standard assay, the resulting micelles are stable with respect to time. To these micelle systems additional calcium was added in increments of 0.02 *M* (final concentration) at intervals of 15 min. The results after assay are shown in Figure 6 for initial ratios of 6, 8, 10, and 12. As expected, at 0.02 *M* calcium considerable precipitate was present only in the system starting at an initial ratio of 12. For comparison, Figure 6 includes the typical curve of Figure 1 for single aliquot addition of calcium at an initial ratio of 10. While the latter falls rapidly to supernatant protein levels below 3 mg./ml. at 0.08 *M* calcium, post-peak incremental addition of calcium produces relatively little instability, for any of the initial conditions, until 0.2 *M* is reached. For example, at 0.08 *M* calcium, single aliquot addition to a solution having an initial ratio of 10 gives a stabilization ratio of 2 while post-peak incremental addition gives a stabilization ratio of 8. Beyond 0.2 *M* calcium, instability is apparent.

On increasing the calcium concentration from 0.02 to 0.2 *M*, although little or no additional precipitate may form, the systems undergo marked structural changes. For an initial ratio of 5 and 10 mg./ml. of α_s-casein, while τ_n may change somewhat (from about 3.7 to 5.7 during 5 hr.), the visual aspect of the system suggests a coarsening of micelles. More revealing are values of micelle solvation and final supernatant protein after ultracentrifugation. The values for micelle solvation are 3.7 ml. of solvent/ml. of protein for the micelle system at 0.02 *M* calcium (see Table II), 3.3 just after increasing the calcium to 0.2 *M*, and 2.8 after 5 hr. Supernatant protein is about 2.2 mg./ml. at 0.02 *M* calcium (see Table II). At 0.2 *M* calcium it immediately decreased to 1.2 but rose to 1.4 after 5 hr.

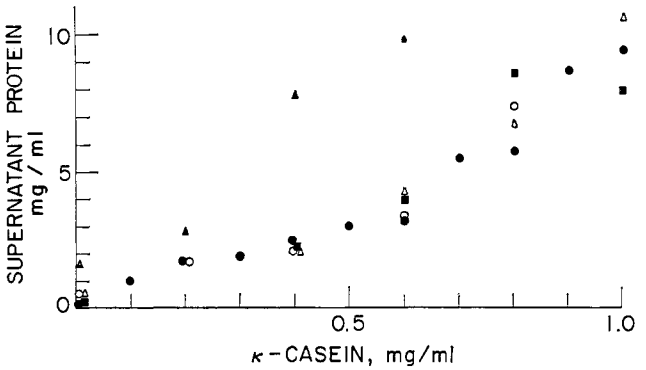

Figure 7. Assay for κ-casein. Supernatant protein, resulting from single aliquot addition of calcium to 0.015 *M*, is plotted as a function of added κ-casein concentration for solutions containing initially 10 mg./ml. of α_s-casein or 10 mg./ml. of an unknown preparation. The symbols are ● for pure α_s-casein; ■ for α_s-casein precipitated at 0.006 *M* $CaCl_2$; O for α_s-casein precipitated at 0.08 *M* $CaCl_2$; △ for a precipitate obtained at 0.006 *M* $CaCl_2$ from a solution initially containing 10 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein; and ▲ for a precipitate obtained at 0.08 *M* $CaCl_2$ from a solution containing initially 10 mg./ml. of α_s- and 1 mg./ml. of κ-casein.

Effect of Adding α_s-Casein to a Micelle System. Attention has been directed to the stability of the micelle system at calcium concentrations in the region of the peak and at initial ratios near 5 (see Figures 2 and 3). If such a system is established, does it retain a capacity to stabilize additional α_s-casein? That it does is shown as follows: A stable micelle system was formed at initial ratio 5 in 0.02 *M* calcium and standard KCl buffer. The α_s-casein concentration was 5.5 mg./ml. To this was added with rapid stirring an α_s-casein solution which increased the volume by 10% but doubled the total α_s-casein concentration; thus α_s-casein was added to increase the final level by 5 mg./ml. Systems were then allowed to equilibrate and were assayed. Of the added α_s-casein, 1.5 mg./ml. appeared as stable micelles. The final stabilization ratio was then 6.5. It should be noted that if calcium had been added as a single aliquot to a system containing 10 mg. of α_s-casein and 1 mg. of κ-casein per ml., stabilization would have been complete (Figure 2).

Characteristics of Precipitates. Precipitates which form in the peak and post-peak regions differ from precipitates of calcium α_s-caseinate.

One important difference is their content of κ-casein. Experiments were performed in which the precipitates from solutions containing initially 10 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein were harvested at 0.006 and 0.08 *M* calcium, redissolved, and analyzed for the presence of κ-casein, using the assay described under Methods. The result of one of these experiments is illustrated in Figure 7. The solid squares represent data for a precipitate obtained at 0.006 *M* calcium (which behaves like pure α_s-casein and is included as a control[3]). The solid triangles represent data for a precipitate obtained at 0.08 *M* calcium. The solid triangles are shifted to the left by 0.3 mg./ml. of κ-casein, indicating the presence of approximately 3% of this protein. This shift was found in four similar experiments.

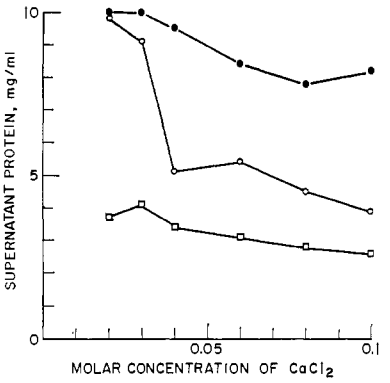

Figure 8. Stabilization behavior of redissolved top, ●, middle, O, and bottom, □, sections of First Cycle casein pellets obtained after centrifuging for 60 min. at 20,000 r.p.m. The First Cycle casein micelle system was formed at 0.02 *M* calcium. Supernatant protein is plotted *vs.* calcium concentration for initial concentrations of 10 O.D. units/ml. Calcium was added in single aliquots throughout.

The bulk and surface characteristics of peak and post-peak precipitates and of the particles which form during attempts to solubilize calcium α_s-caseinate precipitates by κ-casein, and in fact of all precipitates formed in the presence of or in contact with κ-casein at calcium concentrations at or above peak levels, are clearly different from the bulk and surface characteristics of calcium α_s-caseinate precipitate. Particles of the latter rapidly adhere to each other, to glass, or to Teflon to form transparent masses while those which form in the presence of, or are exposed to, κ-casein are to a large extent nonadherent to each other or to glass or Teflon surfaces and yield opaque masses on centrifugation.

For example, a system containing, per ml., 15 mg. of α_s-casein and 1 mg. of κ-casein in standard KCl buffer was made 0.02 *M* in calcium at 37°. As expected, most of the protein appeared as an opaque pellet under the conditions of the assay procedure. The supernatant was decanted and all micelles removed by ultracentrifugation. The resulting clear fluid was now added to the tube containing the assay precipitate. At 37° gentle rotation was all that was required to resuspend most of the precipitate. When quiescent, the particles in suspension settled within a few minutes. Rotation, however, again produced a suspension. This cycle was repeated several times. The transparent precipitate of pure calcium α_s-caseinate will not resuspend. The surface characteristics of peak and post-peak precipitates are in fact similar to those expected of micelles.

These observations on the characteristics of precipitates introduce questions concerning the meaning of the word "micelle." At this stage they direct attention to the relationship between the state of a system and the assay procedure. It is clear that assay supernatant protein includes all protein present in particles too small either to sediment appreciably or to have time to become part of the compacted centrifugate. Increasing the assay gravitational field will progressively increase centrifugate until all micelles (and eventually soluble protein) would be included as such. Increasing assay centrifugation time need not act in the same way, for after a long time all particles must be distributed according to a proper expression of the equilibrium equation for centrifugation.[18] Thus an increase in field reduces the size of a particle which will pack in the centrifugate with negligible supernatant concentration. An infinite number of assay procedures is possible.

The assay procedure given under Methods[3] was chosen on the basis of the following: First, a field of $\sim 400 \times g$ for 1 min. is sufficient to sediment calcium α_s-caseinate precipitate to give a clear supernatant; second, negligible centrifugate is given under conditions for complete stabilization, but under other conditions the centrifugate which forms under assay conditions will also form, for the most part, under $1 \times g$ if given sufficient time, suggesting that precipitate be equated with assay centrifugate; third, when precipitate appears the amount seems to be largely independent of assay centrifugation time. The latter two observations indicate a gap in size between assay centrifugable and noncentrifugable particles.

Size Distribution. Micelle Size and Composition. Sullivan, *et al.*,[19] have shown for the population of micelles present in skim milk that, as the micelle size increases, its sialic acid (κ-casein) content decreases. We have confirmed these important observations and, to eliminate the possibility that a size–composition relationship is due to some aspect of the synthetic mechanisms in the animal, we have examined First Cycle casein.

First Cycle casein at approximately 25 O.D. units per ml. was made 0.02 *M* in calcium by single aliquot addition at 37°. The micelles were centrifuged in the SW 25 rotor at 37° and 20,000 r.p.m. for 60 min. The resulting three pellets were divided into top, center, and bottom sections and comparable sections were combined, brought into solution with potassium citrate, and dialyzed exhaustively against standard KCl buffer. Conditions were chosen to give concentrations in excess of 10 O.D. units per ml. Aliquots were than diluted with dialysate to this level and supernatant protein was examined as a function of calcium concentration at 0.02 *M* and above. Figure 8 gives the results. At all calcium concentrations the degree of stabilization was lowest for the pellet bottom section, intermediate for the center section, and maximum for the top section. Comparison of Figure 8 with the data for mixtures of α_s- and κ-caseins (Figures 2 and 3) suggests that the top section behaves as though it had an initial ratio of approximately 5 and the center and bottom sections as though they had initial ratios of about 10 and 20, respectively.

Size Distribution and Initial Ratio. Initial ratio was examined by centrifugation as follows: Three micelle systems at initial ratios of 2, 4, and 8 were prepared in standard KCl buffer at 0.02 *M* calcium. These systems contained: first, 5 mg./ml. of α_s-casein and 2.5 mg./ml. of κ-casein; second, 5 mg./ml. of α_s-casein and 1.25 mg./ml. of κ-casein; and third, 5 mg./ml. of α_s-casein and 0.62 mg./ml. of κ-casein. The turbidities of these systems increased with increasing initial ratio. Five milliliters of each system was sequentially centrifuged in a SW 39 rotor. After each centrifugation the supernatants were drained into clean centrifuge tubes

(18) H. Fujita, "Mathematical Theory of Sedimentation Analysis," Academic Press Inc., New York, N. Y., 1962, p. 129.
(19) R. A. Sullivan, M. M. Fitzpatrick, and E. K. Stanton, *Nature*, 183, 616 (1959).

and subjected to the next centrifugation. Pellets were dissolved in potassium citrate and protein content was determined. Table I gives the results. The first two columns give successive centrifugation speeds and times. The last three columns give the protein content of the pellets for the initial ratios of 2, 4, and 8. Recovery, including the protein in the final supernatant, was essentially complete. It is obvious that as the initial ratio increases the micelle population is shifted toward more easily centrifuged micelles, thus probably to larger micelles. The conditions of centrifugation and the fact that we are dealing with a polydisperse system make it difficult to associate the conditions of pellet formation with a particular micelle range.

Table I

Centrifugation: Speed, r.p.m. 10^{-3}	Centrifugation: Time, hr.	Pellet protein, mg., for R_i[a] of: 2	4	8
5	5	0.0	0.4	3.4
10	5	1.0	2.1	6.3
15	5	2.4	4.0	6.8
22	5	6.6	6.2	6.4
30	5	7.2	8.5	2.6
30	15	6.6	5.0	1.3
Total pellet, mg.		23.8	26.2	26.8
Total final supernate, mg.		15.6	4.9	2.3
Total, mg.		39.4	31.1	29.1
Initial, mg.		37.4	31.2	28.1
Supernatant protein after the above centrifugation sequence, mg./ml.		3.1	1	0.46

[a] R_i = initial ratio.

The last row of Table I gives final supernatant protein concentrations. Significant supernatant protein concentrations are always obtained, even after removal of micelles by centrifugation at 30,000 r.p.m. (SW 39) for 1 hr. Supernatant proteins have been harvested after the latter centrifugation from systems having initial ratios of 10 and 5 and calcium concentrations of 0.02 and 0.08 *M*, respectively. On starch gel electrophoresis these supernatant proteins were found to contain α_s- and κ-caseins. The band intensities suggested that the α_s/κ weight ratio was low.

Micelle Distribution and Concentration. Turbidity was used as an index of average micelle properties (see Methods). Turbidities may be measured either on aliquots obtained by diluting a system of preformed micelles or on aliquots of original protein solution diluted to different concentrations before calcium addition. Further, in both series, turbidities may be studied easily with respect to time. A calcium concentration in the region of the peak, 0.02 *M*, was chosen to avoid precipitate formation.

A preformed micelle system was first examined. The micelle system, in standard KCl buffer and made by single aliquot addition of calcium to 0.02 *M*, contained 4.5 mg./ml. of α_s-casein and 0.9 mg./ml. of κ-casein. The initial ratio was 5. This system was diluted sequentially by a factor of 2, with standard KCl buffer containing 0.017 *M* calcium, until the final aliquot had one-sixty-fourth of the original concentration. Turbidities were determined immediately after each dilution and again after a period of 5 hr. The turbidities immediately determined lay, within experimental error, on a straight line passing through the origin and with $\tau_n = 1.6$. After 5 hr. all turbidities had increased (by about 10%).

A comparable series was made with the same preparations of α_s- and κ-caseins except that calcium was added after dilution. Initial turbidities, determined after 30 min., were the same as the initial turbidities given above. Again there was a similar slow increase in turbidity with time.

The two series just described suggest that a particular micelle distribution is established largely independently of concentration over the concentration range used. It can also be shown that a concentration range can be chosen so that the micelle distribution is biased toward increasing τ_n as the concentration increases. For example, at an initial ratio of 5, the same protein preparations as used above, and 29 mg./ml. of α_s-casein, τ_n was 3.6.

A concentration dependence is also revealed at unit ratio. Mixtures of α_s- and κ-casein to give individual protein component concentrations of 0.49, 0.99, 1.9, 3.75, 7.5, and 15 mg./ml., in standard KCl buffer at 37°, were made 0.02 *M* in calcium. After 30 min. each was diluted, at 37°, with an equal volume of standard KCl buffer containing 0.017 *M* calcium. Turbidities were measured at these concentrations over a period of 30 hr. and then after dilution of the last three samples to the concentration of the third over an additional period of 15 hr. In this set there was little change of τ_n with time for any system. For the last four systems at 1.7 mg./ml. total protein τ_n was 0.24, 0.37, 0.48, and 0.58. For the first three more dilute samples, τ_n was 0.24.

A concentration dependence of assay centrifugate in the region of the plateau has been described. Systems can be chosen, for example, where no precipitate forms: an initial ratio of 5, 0.08 *M* calcium, and an initial α_s-casein concentration of 5 mg./ml. or below (Figure 3b). For similar conditions at α_s-casein concentrations of 10 and 20 mg./ml. assay centrifugates amounting to about 25 and 60% of the initial α_s-casein appear. As noted above these centrifugates contain κ-casein.

Micelle Solvation. A common observation after ultracentrifugation of casein micelles is that the pellet volume is several times greater than the volume of its protein content. Information concerning solvation has been obtained by examining pellet ultracentrifugates of micelles, of calcium κ-caseinate, and of calcium α_s-caseinate precipitate.

Calcium α_s-caseinate at 10 mg./ml. in standard KCl buffer was precipitated directly in a polyethylene tube by adding calcium to give 0.02 *M*. The tube was centrifuged in the SW 39 rotor at 37° and 35,000 r.p.m. for 30 min. The tube was then drained for 5 min. and the pellet end cut off and weighed. The pellet was dissolved and its protein content obtained from the optical density of the resulting solution. The tube end was recovered, washed, and weighed, and the pellet weight was obtained by subtraction.

Calcium κ-caseinate at 10 mg./ml. and several micelle systems at 10 mg. of α_s-casein/ml. but at different initial ratios were examined using this technique,

Table II. Micelle Solvation

Initial ratio	Time after Ca addn., hr.	Pellet: S	Pellet: solvent volume / protein volume, Sphere packing	No. of detn.	Supernatant protein, mg./ml.	Total recovery, %	τ_n, O.D./mg.
1	5	4.5	3.1	1	11.5	98	0.42
2	5	5.0	3.4	1	4.9	100	0.45
3	0–6	4.5	3.1	3	3.7	100	2.3
5	0–6	3.7	2.5	4			
5	0	4.3	2.9	1	1.9	92	3.5
5	3	3.9	2.6	1	2.2	95	4.6
5	6	3.4	2.2	1	2.9	102	5.1
8	0–6	3.3	2.2	3	0.7	94	4.2
20	4	2.8	1.8	3	0.4	97	Precipitate
κ	1	5.9	(4.1)	1	0.44	94	
α_s	1	2.2		1	0.06	100	
α_s	0.5	2.1		1	0.07	99	
α_s	1	2.2		1	0.07	100	

except that the aliquots for centrifugation were drawn from a larger volume. Table II gives the results of experiments carried out on particular lots of κ- and α_s-caseins: The first two columns give the initial ratio and the time after calcium addition when ultracentrifugation was initiated. The next two columns give the ratio, in the pellet, of solvent volume to protein volume assuming solvent and protein densities of 1.0 and 1.3, respectively. These values give minimum values of solvation. Column 3 is the observed solvation and column 4 is calculated from this on the basis that pellets contain close-packed micelle spheres of constant diameter embedded in supernatant. The remaining columns are self-explanatory. It is evident that the micelle is solvated to a greater extent than is calcium α_s-caseinate, to a smaller extent than is calcium κ-caseinate, and that micelle solvation decreases progressively with increasing initial ratio, approaching that of calcium α_s-caseinate. As the initial ratio increases supernatant protein decreases and τ_n increases, as expected.

In Table II results have been combined where differences in solvation values were less than 5%. The center section, for an initial ratio of 5, refers to an experiment where turbidity increased with time. In this experiment, solvation appears to decrease and final supernatant protein to increase with time.

Total recovery is often less than 100%. An important source of error is the efficiency with which supernatant, and particularly any concentrated layer lying near the pellet surface, can be decanted into tubes from which supernatant protein is determined.

Turbidity and Time. It has been observed that sometimes the turbidity of a micelle system is least just after calcium addition and that it increases over a period of several hours. The initial turbidity, the extent of increase (turbidity may remain unchanged or even double in 24 hr.), and the time course of increase are apparently sensitive to uncontrolled details of the experimental procedure such as mixing and thus are difficult to reproduce quantitatively. However, the turbidity stabilizes at some final value. Comparison of a micelle system before and after dilution gave some indication that the fractional change is greatest for the undiluted system. Thus, although systems are stable to the supernatant protein assay, internal rearrangements may take place over a period of several hours. The final turbidity is clearly path dependent. Sources of turbidity change have been considered (see Methods).

Discussion

Any model proposed for the mechanism of formation and structure of casein micelles must accommodate certain experimental results. The most important of these appear to be the following: (A) Prior to micelle formation at 37° there are present free calcium α_s-caseinate and a low weight ratio calcium α_s–κ-caseinate interaction product. (B) For stability, micelles require a minimum level of calcium which is greater than that required to precipitate the free α_s-casein present. (C) The more centrifugable (larger) the micelle the lower its weight fractional content of κ-casein. (D) Precipitates formed in the presence of κ-casein and at calcium concentrations sufficient for micelle stability are different from calcium α_s-caseinate precipitates in being nonadherent and compacting to give white opaque pellets. These precipitates contain small amounts of κ-casein. Calcium α_s-caseinate precipitates treated with κ-casein become nonadherent. (E) Micelles are stable with respect to the close approach induced by sedimentation into a pellet. (F) In all cases micelles have a size distribution which depends strongly on the initial ratio, the calcium concentration on single aliquot addition, and the initial protein concentration. The apparent final states of the systems are dependent on the path. Path dependencies were found for assay supernatant protein, supernatant protein after ultracentrifugation, and a wide variety of micelle–precipitate combinations obtained either by single aliquot or incremental addition of calcium. (G) Complete stabilization in the region of the peak can be achieved at all initial ratios up to 10 and micelle populations stable with respect to assay conditions may have weight ratios in excess of 10. (H) The size distribution of micelles can be altered by the addition of κ-casein and probably by the addition of calcium. Size changes induced by κ-casein addition occur rapidly (minutes) while those attending calcium addition take hours. κ-Casein has some capacity to solubilize calcium α_s-caseinate precipitate but the resulting stabilization ratios are small (0.5–2) compared to stabilization ratios easily attained in the region of the peak. (I) Stabilizing capacity is available in some micelle systems after the systems have

come to apparent final states. (J) A micelle distribution, once formed, is relatively stable to dilution with a buffer containing an appropriate calcium concentration. (K) Micelles are highly solvated and the solvation decreases as the initial ratio increases. (L) Although micelles require the presence of α_s- and κ-caseins, they can incorporate variable amounts of β-casein.

Micelles are expected to fall into one of the categories of (a) single phase particles, (b) large chemical compounds, or (c) structures having a composition which changes in going from the surface to the center.

A single-phase particle is one in which average composition is uniform throughout. Where surface orientation occurs, such orientation will take place spontaneously when a new surface is exposed by subdivision of the original particle. It seems unlikely that systems of single-phase micelles could be in equilibrium. The variety of chemical compositions present in any single system would require violation of the phase rule. If this were not sufficient, in addition all micelles would need to have zero surface energy and be in equilibrium with the same levels of constituent components.

If the micelles were nonequilibrium single-phase particles having positive surface energies, stability would depend on prevention of contact coupled with a sufficiently slow exchange of individual components so that the lifetime of the system is long compared to the duration of the experiment. It would then be expected that artificially bringing micelles into surface contact would destabilize the system with precipitate formation. As seen, pellet centrifugates can easily re-establish micelles.

Soap micelle systems have been treated with partial success as single-phase systems.[20] In such systems micelles are composed of a single type of molecule and all constituent molecules are at the surface. It seems unlikely that the casein micelle system could conform to this type of model since micelles are spherical and the larger micelles have radii several times greater than the dimensions of their constituent subunits. The results listed at the start of this discussion would have to be accounted for. Particularly difficult would be those associated with stabilization ratio and micelle composition.

The last two observations also argue effectively against a model which assumes micelles to be large chemical compounds. A variety of molecules and particles such as hemoglobin,[21] tobacco mosaic virus,[22] or hemocyanin[23] are made up of subunits of different structure. Subunit association in these systems appears to be limited by the formation of a single kind of association product in which interaction specificity uniquely defines geometry and composition.

It seems most likely that micelles have a composition which changes in going from the surface to the center. In view of the solubility characteristics of α_s- and κ-caseins in the presence of calcium, the composition gradient of choice is a κ-casein-rich surface and an α_s-casein-rich core. The simplest structure that would conform to this hypothesis is one having a pure calcium α_s-caseinate core covered by a uniform coat composed of a low weight ratio calcium α_s–κ-caseinate interaction product.[24] Obviously calcium α_s-caseinate in the core need not be in contact, therefore in exchange, with the environment. In this model the coat subunits are required to have certain interaction properties. That portion of the subunit surface exposed has little tendency to interact with itself or other constituent proteins. The subunits have internally a strong interaction with core calcium α_s-caseinate and probably a lateral preference for each other. Components in the surface (calcium, κ-casein, and a subunit calcium α_s–κ-caseinate complex) are in exchange with similar components in solution. As the core is reduced a limiting micelle composed of calcium α_s–κ-caseinate coat material is approached.

Kinetically, the addition of calcium to a solution containing α_s- and κ-caseins initiates at least two sets of reactions: The first leads to the precipitation of calcium α_s-caseinate and the second to a coating process that produces stable micelles. The simplest evident mechanism is that the development of calcium α_s-caseinate particles and the acquisition of a coat are random processes.

Certain experimental results are readily accounted for by this model as described so far, while other results suggest the addition of more detailed specifications.

The model readily accepts free α_s-casein and a low weight ratio interactant as the state of the system just after calcium addition (A). The calcium requirements (B) result from the observations that calcium α_s-caseinate precipitation in the region of the dip can be obtained either by adding calcium to a protein mixture or by diluting a preformed micelle system,[3] both to a calcium concentration in the region of the dip. Upon dilution the coat becomes unstable and goes into solution. Cores then precipitate to establish the appropriate level of calcium α_s-caseinate solubility. A clear inference is that the calcium dependency of coat formation must be greater than that of calcium α_s-caseinate precipitation.

The relationship between size and chemical composition (C) and precipitate characteristics (D) are directly accounted for.

Stability to close approach (E) is also directly accounted for given one additional provision. Packing at the highest centrifugal fields may result in distortion and/or alterations in solvation, both of which by this model may lead to larger micelles or particles unstable to the assay centrifugation.

The kinetics of micelle formation readily account for the dependency, for example, of micelle size and micelle–precipitate distributions on increased initial ratio, increased protein concentration, and increased post-peak calcium concentration *via* single aliquot addition (F). In each case an increase is considered differentially to favor calcium α_s-caseinate particle formation over coat formation. There results a shift toward larger micelles and an increasing fraction of these become unstable to assay centrifugation. The limit is the precipitation of calcium α_s-caseinate prior to initia-

(20) K. Shinoda and E. Hutchinson, *J. Phys. Chem.*, **66**, 577 (1962).
(21) A. F. Cullis, H. Muirhead, M. F. Perutz, and M. G. Rossmann, *Proc. Roy. Soc.* (London), **A265**, 161 (1962).
(22) A. Klug and D. L. D. Caspar, *Advan. Virus Res.*, 7, 225 (1960).
(23) E. F. J. van Bruggen, E. H. Wiebenga, and M. Gruber, *J. Mol. Biol.*, 4, 1 (1962).

(24) The coat could be pure calcium κ-caseinate, but this appears unattractive as will be evident from a discussion of the effects of κ-casein on calcium α_s-caseinate precipitates and on micelle systems, the retention of excess stabilizing capacity, and the source of the instability of the micelle system to added calcium.

tion of coat formation. In this view there is no qualitative difference between a particle small enough to be coated and give rise to a stable micelle and a particle so large that it settles readily under unit gravitational field. Kinetically, complete stabilization (G) corresponds to the virtual absence of particles large enough to be sedimented during assay centrifugation.

In considerations of size distribution and final state (F) as affected by component concentration, it must be recognized that kinetic and nonkinetic effects both depend upon the relative amounts of some components. For example, the rate of coat formation and the maximum potential coat area are both related to the amount of κ-casein.

To be introduced next are detailed specifications required by the results obtained when the micelle environment is altered, for example, by the addition of κ-casein or calcium. Restrictions must be placed on the concentrations of certain components in an environment in contact with micelles. However, it will be apparent that a particular micelle distribution does not necessarily require a particular environment.

The addition of κ-casein may produce a rapid decrease in micelle size (H). The mechanism proposed now is that calcium κ-caseinate enters the coat of core micelles but later leaves as a calcium α_s–κ-caseinate complex.[25] This interpretation is supported primarily by the ability of calcium κ-caseinate to solubilize either calcium α_s-caseinate precipitates or assay precipitates produced at higher initial ratio, and secondarily by the fact that the supernatant protein after ultracentrifugation increases with decreasing initial ratio.

Certain apparently stable micelle systems retain a capacity to stabilize additional α_s-casein (I). Thus, during the kinetics of micelle formation, not all κ-casein is utilized as coat material for core micelles; some must remain as available coating material. A question immediately comes to mind. If there is excess stabilizing capacity, why does this not reduce the size of the micelles with which it is in contact, as does added κ-casein? The conclusion is that the coat material is not pure κ-casein. It seems reasonable to assume that the coat material can stabilize a free calcium α_s-caseinate surface but cannot enter and expand the coat layer of a core micelle. The effect of κ-caseinate is ascribed to its capacity to extract calcium α_s-caseinate from micelles. It is obvious from this that there is a maximum low level of free calcium κ-caseinate.

If there were no micelle resistance to distortion, excess stabilizing capacity should not persist and micelles would be expected to have a variety of shapes. Only spherical micelles have been found by others[26] or by us.[8] A lack of surface reactivity, as required by this model, and a preferred spherical shape would be accommodated by a tension or its equivalent at the core–coat interface.

The addition of calcium to a micelle system probably increases micelle size (H). This possibility suggests that the coat has its maximum stability at calcium concentrations in the region of the peak (Figures 2 and 6). Assuming that this is the case, the addition of sufficient calcium must produce a surface instability such that coat material is removed, revealing calcium α_s-caseinate surface. Micelle coalescence then follows until complete coverage is again attained and there is established a new coat–environment relationship consistent with micelle stability. Assume that the coat is constructed of identical subunits of calcium α_s–κ-caseinate. Coat instability would result if a coat subunit component is involved in a reaction to produce noncoat material and this reaction has a higher calcium dependence than the coating reaction. Relative equilibrium constants would determine the calcium concentration range over which each of these reactions is effective and where they compete with each other. It is obvious that a competitive reaction for a coat subunit component might be the formation of a calcium α_s–κ-caseinate interaction product having a lower weight ratio than the coat subunit (possibly this is pure calcium κ-caseinate).

Retaining the assumption that the coat subunit has a preferred α_s/κ weight ratio, we examine the environment in contact with micelles. It is apparent that the level of free calcium α_s–caseinate must be negligible compared to other protein components. The latter are expected to occur predominantly as coat subunits and as calcium α_s-κ-caseinate complexes having weight ratios lower than that of the coat complex and including small amounts of calcium κ-caseinate. The kinetic aspect of this model, however, is consistent with the formation of some small particles, possibly to be classified as micelles, which remain in the supernatant even after ultracentrifugation and which have weight ratios higher than that of the coat subunit. The question is: To what extent is the coat subunit weight ratio revealed by equilibrium levels associated with (a) stabilization ratios after calcium κ-caseinate solubilization of assay precipitates or of calcium α_s-caseinate precipitate; (b) stabilization ratios of calcium α_s-caseinate at low calcium concentrations in the descending limb of the dip, either by single aliquot or incremental addition of calcium; and (c) stabilization ratios of the supernatant protein after incremental addition of calcium to concentrations in the region of the peak. To have meaning these stabilization ratios should be obtained in the presence of coated calcium α_s-caseinate precipitate or core micelles under conditions where it can be assumed that negligible κ-casein is included in the precipitate. Ideally, there must be no core micelles in the assay supernatant or other fluid used to obtain stabilization ratios. The level of calcium should be one giving maximum coat stability (0.02 *M* calcium). Stabilization ratios ranging from 0.6 to 3.0 have been obtained under sets of conditions each to some extent satisfying the above requirements. Core micelle formation certainly occurs under the conditions where the higher stabilization ratios were obtained (vigorous stirring during the solubilization of calcium α_s-caseinate). In this respect, however, attention is directed to final stabilization ratios attending incremental addition of calcium to concentrations in the region of the peak (see Figure 3 of ref. 3). At the bottom of the dip where appreciable levels of calcium α_s-caseinate are still in solution, the coating process is in

(25) A less attractive alternative is that calcium κ-caseinate enters the coat and increases the coat area. The resulting distortion produces an energetically unfavorable situation which is resolved by fragmentation. This alternative and that suggested in the text require an energetic mechanism for maintaining spherical micelle shape.

(26) H. Nitschmann, *Helv. Chim. Acta*, **32**, 1258 (1949); H. Hostettler and K. Imhof, *Milchwissenschaft*, **6**, 351 (1951).

operation and final stabilization ratios at 0.01 M calcium must thus include core micelles. The values of $\sim$2 obtained in these experiments do, however, suggest an upper limit to a coat subunit weight ratio. Under conditions where core micelles would not be expected to contribute to stabilization ratios, but where equilibrium might not have been attained, stabilization ratios are as follows: 0.9 and 0.8 for solubilization of assay precipitates, 0.6 and 0.6 for solubilization of calcium α_s-caseinate precipitate, and 1 and 1.3 for stabilization on incremental addition of calcium during the descending limb of the dip. We suspect that these lower weight ratios cover a range which includes the coat subunit.

A source of coat material, which might be a reversibly dissociable coreless micelle or other coat subunit association product, would account for the apparent stability of micelle distributions to dilution (J). Such association products would serve as a buffer to maintain the necessary level of coat material in the environment. In addition, equilibrium levels of coat material might be sufficiently low so that dilution produces so slight a micelle coat dissociation that micelle characteristics are not affected. This is suggested by the extent and equivalence of resuspension of micelle pellets either in their supernatants or in standard KCl buffer containing appropriate calcium.

The degree of solvation of micelles and the variation in solvation with initial ratio (K) is expected from the characteristics of calcium α_s- and calcium κ-caseinates. This model readily accommodates these results; the high level of solvation associated with the coat would contribute to stability to close approach. The main difficulties with studies of solvation using pellet centrifugates are the extent to which supernatant is included in the pellet and the extent to which micelles are desolvated by the centrifugal field. Data, in addition to Table II, suggest that there are concomitant problems. More refined methods are required.

While mixtures of pure κ-casein and β-casein will not form micelles in any way comparable to those of mixtures of κ-casein and α_s-casein, considerable amounts of β-casein, at least up to 30% of the α_s-casein plus β-casein moiety, are readily incorporated. First Cycle casein behaves much like an α_s–κ-casein mixture of weight ratio 7. At 37° the solubility of calcium β-caseinate is comparable to that of calcium α_s-caseinate.[6] In this model the coating of a calcium α_s–β-caseinate core is readily accepted provided the β-casein fraction is not so high as to prevent formation of an effective coat layer.

There is a feature of this model which has additional meaning: κ-casein is placed at the surface where it can be attacked easily and quantitatively by rennin. Since carbohydrate is released into solution by rennin action,[27] the carbohydrate substituent of κ-casein would be placed near the micelle surface and might on account of its solvation characteristics be responsible for the stability of micelles to close approach. The placement of κ-casein at the surface also permits alteration of κ-casein without micelle dissociation, a circumstance which would allow rennin clotting of the extremely stable micelles formed by Cu, Ni, Co, Mn, Cd, and Zn.[17] As has been pointed out, κ-casein is the only casein component altered during the time required for rennin coagulation.[6] This model places α_s- and β-caseins so that they are not in contact with the environment. In addition, rennin action to produce para-α_s- and para-β-caseins is not only slower by a factor of $\sim 10^3$ but the products are nonprecipitable by calcium.[6]

(27) H. Nitschmann and R. Henzi, *Helv. Chim. Acta*, 42, 1985 (1957); H. Nitschmann and R. Beebe, *Chimia* (Aarau), 14, 318 (1960); P. Jolles, C. Alais, and J. Jolles, *Biochim. Biophys. Acta*, 51, 309 (1961); J. Garnier, *Ann. Biol. animale Biochim. Biophys.*, 3, 71 (1963).

Dismutation Reactions of Nucleoside Polyphosphates. I. General Features of the Reaction

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Contribution No. 22 from The Syntex Institute for Molecular Biology, Stanford Industrial Park, Palo Alto, California. Received January 22, 1965

Adenosine 5′-triphosphate dissolved as its tributylamine salt in anhydrous pyridine at room temperature undergoes a rapid dismutation reaction. The main products of this reaction have been characterized as a homologous series of adenosine 5′-polyphosphates containing up to seven phosphate groups. A second minor series of products has been isolated and characterized as α,ω-di(adenosine-5′) polyphosphates. Similar types of products arise upon storage of anhydrous pyridine (or mixed solvents containing pyridine) solutions of adenosine 5′-di- or -tetraphosphate and of p-nitrobenzyl triphosphate. The presence of excess ortho- or pyrophosphate anions prevents the formation of products of increased polyphosphate chain length and leads to the accumulation of adenosine 5′-mono- and -diphosphates. A similar effect is produced by the addition of intermediate amounts of water. The stability of nucleoside polyphosphates in solvents such as dimethyl sulfoxide permits syntheses that would be extremely difficult in pyridine.

(1) (a) Syntex Postdoctoral Fellow, 1961–1962. (b) Syntex Postdoctoral Fellow, 1963–1964, supported in part by the "Stiftung für Stipendien auf dem Gebiete der Chemie" (Switzerland), whose aid is gratefully acknowledged.