Casein Micelles. Formation and Structure. I[1]

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Casein interactions which take place prior to and during micelle formation have been studied using either a variety of mixtures of α_s- and κ-caseins or First Cycle casein or Solubilized Skim Milk. Techniques have involved analytical and preparative ultracentrifugation and, for systems to which calcium was added, measurements of supernatant protein at 37° after assay centrifugation for 1 min. at 400g. In the absence of calcium at pH 7 and 2°, α_s- and κ-caseins show little or no tendency to interact and interaction products will not develop after urea or alkaline treatment. At 20°, while interaction products are normally absent, they appear after urea or alkaline treatment. At 37° a variety of condition-sensitive interaction products normally exist. The addition of calcium to give low concentrations produces no visible effect on solutions at pH 7 containing α_s-casein or mixtures of α_s- and κ-caseins. Depending on protein concentration, precipitation of calcium α_s-caseinate is initiated from both at the same calcium concentration: at 0.004 M calcium for 5 mg./ml. of α_s-casein and at 0.005 M calcium for 10 mg./ml. of α_s-casein. Precipitation from mixtures is thereafter progressively retarded by the presence of κ-casein and to an extent corresponding to a weight ratio near unity just before micelle formation is initiated. Thus, the only interaction products that can survive the addition of small amounts of calcium, or which are formed just prior to micelle formation, must have weight ratios near unity. That the behavior of α_s–κ-casein mixtures is not an artifact of preparative procedures is shown by a similar behavior on the part of either First Cycle casein or of Solubilized Skim Milk. The addition of calcium, in single aliquots to calcium-free mixtures of α_s- and κ-caseins, to give concentrations between 0.007 (depending on protein concentration) and 0.02 M leads to increasing micelle formation and complete stabilization if the initial α_s/κ weight ratio, R_i, is 10 or less. Thus, a pronounced dip, having descending and ascending limbs, appears in the plot of supernatant protein vs. calcium concentration. Progressively above $R_i = 10$ assay centrifugate increases, but the amount of protein remaining as supernatant micelles greatly exceeds the sum of initial κ-casein plus calcium α_s-caseinate solubility. It is apparent from the occurrence of the dip that micelle-forming reactions have a higher calcium dependency than calcium α_s-caseinate precipitation. The dip also presents evidence consistent with low weight ratio interaction products, since the dip decrements as R_i decreases but is small and still apparent at $R_i = 2.5$. If calcium is added incrementally to solutions containing α_s-casein or α_s–κ-casein mixtures at $R_i < 10$ the descending limb of the dip appears as for single aliquot addition (it also appears on dilution of preformed micelles) but with increasing incremental calcium the ascending limb does not appear; the descending limb continues its downward excursion, leveling off at apparent α_s/κ supernatant stabilization weight ratios of 2 to 3, which are to be compared with ratios of 10 to 12 for single aliquot addition of calcium. This and other path dependencies are interpreted to mean that this is a nonequilibrium system under our experimental conditions; the equilibrium state thus cannot be defined. Specificity of interaction does not in itself lead to the formation of α_s–κ-casein complexes in which interaction sites on κ-casein dictate a maximum stoichiometry and by this mechanism a stabilization ratio. Calcium binding may be calculated from the displacement with respect to α_s-casein concentration of plots of calcium α_s-caseinate solubility vs. total calcium. The data are consistent with the binding of seven calcium ions just prior to precipitation with an additional increment of four for the formation of the precipitate.

Introduction

The milk system has excited interest for well over a century: first as a possible source of a uniform protein[3,4] and more recently as representing a colloid of remarkable stability whose development could only result from a hierarchy of specific interactions.[5–7]

It was the pioneering work of Linderstrøm-Lang and his colleagues before 1928[8,9] which demonstrated for the first time that the casein of Hammarsten[4] contained more than one casein component. Fractions obtained by acid–alcohol extraction varied in their response to the addition of calcium ion, from certain minor fractions on which calcium had apparently no effect, to major fractions which on addition of calcium formed a milky colloid, possible showing incipient precipitation. This behavior pattern suggested to Linderstrøm-Lang a fundamental postulate, namely, that soluble components interact with and stabilize less soluble components. Pure fractions were not available to Linderstrøm-Lang and since mixing experiments were not conducted a direct demonstration of the protective effect was not made. Linderstrøm-Lang did, however, predict that a component or components insoluble in the presence of calcium ion would eventually be isolated[8] and suggested further that the clotting enzyme rennin would be found to split out a

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protecting material, allowing the others to precipitate.

Isolation of a component on which calcium had no effect occurred again in 1956. In the meantime more efficient and less objectionable fractionation procedures than those of Linderstrøm-Lang had been introduced by Warner,[10] who designated the major fractions he obtained as α- and β-caseins, corresponding to the electrophoretic patterns of Mellander.[11] The suggestion of protective action persisted. Cherbuliez and Baudet[12,13] found that β-casein was precipitable by calcium, that α-casein formed a colloid in the presence of calcium, and that α-casein was capable of stabilizing β-casein. Clearly, however, in this case α-casein lacked the calcium insensitivity of some of the minor fractions of Linderstrøm-Lang.

More recently and Waugh and von Hippel[5,14] have shown that classical α-casein is made up of two major components, α_s- and κ-caseins. α_s-Caseins are precipitated by calcium[6,15] while κ-casein is calcium insensitive. The latter, which accounts for about 15% of the total casein, is responsible for initiating the events leading to the stabilization of α_s- and β-caseins, which together account for 75–80% of the total casein. Although β-casein is normally always present, the necessary and sufficient requirements for micelle formation have been shown to be α_s-casein, κ-casein, and a divalent cation such as calcium.[7] Apparently most of the interesting properties of the milk system, such as the size distribution of micelles, can be duplicated in the absence of β-casein.

Knowledge concerning the series of interactions which take place prior to and during micelle formation are of paramount importance to our understanding of the system. Our purpose here is to examine the characteristics of mixtures of α_s- and κ-caseins, at a variety of weight ratios and in the absence and presence of calcium, the latter particularly at concentrations below those required to form micelles. These characteristics are compared with similar characteristics of Solubilized Skim Milk and First Cycle casein, the latter containing the family of caseins essentially free of whey protein. The group of studies sheds light on the interactions of α_s- and κ-caseins and establishes certain critical properties which any model for micelle formation and structure must accommodate. The system will be shown to be more complicated than proposed by Waugh and von Hippel.[5–7]

Materials and Methods

Skim Milk. Milk, obtained from individual Guernsey cows, was cooled at once, skimmed by bucket centrifugation at 800 $\times$ g for 45 min., frozen, and stored at $-15°$.

Solubilized Skim Milk was prepared by adding solid potassium citrate to skim milk to give a concentration of 0.2 M. The resulting solution was then dialyzed extensively against 0.07 M KCl or NaCl adjusted to pH 7.2 with NaOH. After dialysis the solution was clarified by centrifugation at 55,000 $\times$ g for 15 min., after which it was frozen and stored at $-15°$.

First Cycle casein, which contains all members of the casein group essentially free of whey protein, was prepared by the oxalate technique of von Hippel and Waugh[14] and by the citrate technique of Waugh and coworkers[15] except that the final dialysis was against 0.2 M KCl. In both cases solutions were frozen and stored at $-15°$.

$\alpha_{s1,2}$-Casein was prepared by the method of Waugh and co-workers.[15]

Fraction S and fraction P (*Second Cycle casein*), the soluble and insoluble fractions obtained by a calcium–temperature splitting procedure, were obtained as described by Waugh and von Hippel.[5] Preparations were frozen and stored at $-15°$.

κ-Casein. Of the three major caseins, κ-casein in our experience has been the least amenable to physical and chemical study. Furthermore, the literature contains conflicting reports concerning its properties.[5,6,16–22] Because of these circumstances and the possibly drastic conditions used in some of the preparative procedures, we have compared κ-casein preparations made according to the following methods. All were found to have similar micelle-forming properties, although from sample to sample their maximum stabilization capacities differ by about 5%.

The starch gel patterns given by alcohol-fractionated and DEAE–cellulose-fractionated κ-caseins are the same and correspond to those of McKenzie and Wake.[16] The ultracentrifuge-fractionated κ-casein had a pattern which suggested the presence of β-casein and some minor components. However, α_s-casein was absent. This latter type of preparation had the lowest stabilizing capacity.

(*a*) *Alcohol-fractionated κ-casein* was made by a modification of the procedure of McKenzie and Wake.[16] The starting material is fraction S dialyzed at 4° against 0.005 M NaCl at pH 7.2. The solution is brought to room temperature, diluted with an equal volume of 95% ethanol, and titrated with 2 M ammonium acetate in 50% ethanol until precipitation is complete. The precipitate is dissolved either in water at pH 9 or in 2 M urea adjusted to pH 7 and dialyzed *vs.* 0.005 M NaCl at pH 7.2. The procedure is repeated if necessary. The final product is freeze dried and stored at $-15°$.

(*b*) *Ultracentrifuge-Fractionated κ-Casein.* This general technique was referred to previously.[6] Fraction S, without removal of calcium, is adjusted to pH 7.2 and centrifuged at 70,000 $\times$ g for 30 min. at room temperature in the No. 30 Spinco rotor. The precipitate is discarded and the supernatant is cooled to 8°, returned to the rotor, and centrifuged for approximately 18 hr. at 8° and 70,000 $\times$ g. Centrifugation produces a pellet, often with a dense layer of protein above it. These two layers are harvested, dissolved in

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100 ml. of distilled water, freeze dried, and stored at $-15°$.

(c) *DEAE-Fractionated κ-Casein.* The starting material for column fractionation is ultracentrifuge-fractionated κ-casein. The procedure is a modification of that first developed by Garnier and Waugh.[23] A DEAE–cellulose column 8.4 cm. in diameter and 5 cm. long is washed alternately with 1-l. volumes of 4.5 *M* urea containing 0.1 *M* NaOH or 0.1 *M* HCl (two cycles), then with 600 ml. of 4.5 *M* urea containing 0.15 *M* imidazole at pH 7, and finally with 4.5 *M* urea containing 0.01 *M* imidazole at pH 7 (buffer I). Approximately 1000 mg. of ultracentrifuge-fractionated κ-casein is delivered in 500 ml. of buffer I at a rate of 25 ml./min. The column is washed with 1 l. of buffer I and 2.5 l. of buffer I containing 0.13 *M* NaCl. Buffer I containing 0.5 *M* NaCl is then used to elute κ-casein. The yield is 30–50% of the initial load. The product is dialysed against 0.02 *M* KCl, freeze dried, and stored at $-15°$.

Protein Concentration. The extinction coefficient, $\epsilon_{1\%,\,280}^{1\text{ cm}}$, of $\alpha_{s1,2}$-casein has been shown to be 10.1[15] and that of κ-casein 10.5 ± 0.5 by Garnier[24] and 9.2 in this laboratory. We have used a value of 10 for both proteins. For First Cycle casein and other systems containing β-casein, whose extinction coefficient is well below 10, concentrations are expressed in terms of optical density units (O.D. units) per ml., where one O.D. unit is equal to the amount of protein which, when dissolved at pH 7 in 1 ml., has unit optical density in a 1-cm. cell at λ 2800 Å.

Standard KCl buffer contains 0.07 *M* KCl and 0.01 *M* imidazole at pH 7.1.

Materials and Methods Previously Described.[15] Among these are chemicals, laboratory distilled water, urea purification, treatment of dialysis tubing, and DEAE–cellulose and its refinement.

Starch Gel Electrophoresis. This method was carried out as described by Poulik[25] and in the presence of urea as described by Wake and Baldwin.[26]

Ultracentrifugation. The Spinco analytical machine was used with its schlieren optical system and a phase plate. Temperature was controlled during a run and all runs were made at 59,780 r.p.m. Two buffer systems have been used and compared: the phosphate buffer of Waugh and von Hippel[5] at $I = 0.15$, containing 5.23 g. of K_2HPO_4, 2.72 g. of KH_2PO_4, and 2.96 g. of KCl per liter of solution; and standard KCl buffer. Our Spinco preparative ultracentrifuge has been further modified for accurate control of temperatures above room temperature. The rotor is monitored by employing a device sensitive mainly to radiation exchange with the rotor rather than with the walls of the chamber or rotor atmosphere. It utilizes the the principle described by Waugh and Yphantis[27] but instead of a thermocouple employs a thermistor and Yellow Springs Model 71 Thermistemp control operating a heater in the vacuum chamber. The rotors referred to in the text were obtained from the Specialized Instruments Corp., Palo Alto, Calif.

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Assay for Supernatant Protein. When calcium is to be added in a single aliquot, 1 ml. of the desired solution in a 15 × 75 mm. serological test tube is placed in a 37° bath and allowed to equilibrate for 10 min. Then 0.111 ml. of $CaCl_2$ solution with a concentration ten times the desired final calcium concentration is added by means of a syringe. A Teflon disk (1 cm. in diameter) is attached to the end of the needle to permit rapid stirring of the solution during calcium addition.[5] Following calcium addition, solutions are allowed to equilibrate for 75 min., the first 60 min. of which they are shaken by being placed in a 37° bath at an angle of 45° and reciprocating at a frequency of 180 c./min. using a stroke of 4 cm. They then stand quiescent for 15 min. Shaking was carried out since in its absence double layered precipitates are sometimes found to form; the upper layer of this precipitate pours off on decanting the supernatant. The tube size (15 × 75 mm.) is dictated by the shaking procedure as the smallest tube in which surface tension does not make agitation difficult. After equilibration the tubes are centrifuged for 1 min. at 400 × *g* in a clinical centrifuge (International Model CL). The supernatant is then decanted and diluted with 0.05 *M* potassium citrate, and the optical density of the resultant clear solution is determined at 2800 Å. A turbidity correction, obtained from the optical density reading at 3200 Å., is used when necessary. This gives the *supernatant protein.*

For incremental addition of calcium, the protein solution (10 to 20 ml.) is placed in a jacketed beaker with 37° water circulating through the jacket. Aliquots of 2 *M* $CaCl_2$ solution to give increments of 0.002 *M* are added with rapid stirring at 15-min. intervals by means of a micrometer syringe. Stirring is continued during the 15-min. interval and then a 1-ml. sample is removed just before the next calcium addition. The 1-ml. samples are centrifuged and assayed as just described.

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 κ-casein concentration is referred to as the *stabilization ratio.* These values are accurate only if the precipitates contain negligible κ-casein (see below). It should be noted that the solubilities determined for calcium α_s-caseinate by this assay should not be construed as the thermodynamic solubility.

Results

Illustrative Behavior of α_s–κ-Casein Mixtures upon Single Aliquot 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. 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

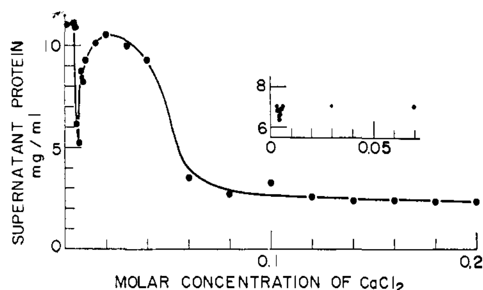

Figure 1. Supernatant protein resulting from single aliquot addition of calcium plotted as a function of $CaCl_2$ concentration for two α_s–κ-casein mixtures in standard KCl buffer at 37°. The main curve represents data for a solution initially containing 10 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein. 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.

decreases rapidly until 0.06 M calcium is reached after which it decrements slowly. In the region of increasing supernatant protein between 0.006 and 0.015 M calcium a reproducible secondary dip is apparent.

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 will be 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 lastly 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 the turbidity of the system until at approximately 0.01 M calcium it has the appearance of milk.

Component Solubility Behavior. κ-Casein. This protein is not precipitated from solution by addition of calcium ion except at very high concentrations, where gel formation or salting out occurs. Such concentrations were never used in the experiments to be described. Apparently even the state of aggregation of κ-casein is essentially unaffected by calcium.[5]

α_s-Casein. Figure 2 illustrates the solubility of calcium α_s-caseinate as a function of calcium concentration in 0.07 M KCl at pH 7. Initial α_s-casein concentrations of 5 and 10 mg./ml. were used. The relative displacement of these two curves along the abscissa indicates calcium binding, a subject which will be discussed below. Up to the calcium concentration which produces precipitation, each solution is clear and beyond this concentration all supernatants are clear.

Comparison of the Dip Region with Calcium α_s-Caseinate Solubility. Single Aliquot Addition of Calcium to α_s-κ-Casein Mixtures. The α_s-casein solubility curve for 10 mg./ml. of initial protein is shown as the lower solid line of Figure 3. The other lines refer to α_s–κ-casein mixtures each containing 10 mg./ml. of α_s-casein and decreasing amounts of κ-casein ranging from 1 to 0.5 mg./ml. (α_s/κ initial ratios of 10 to 20). With

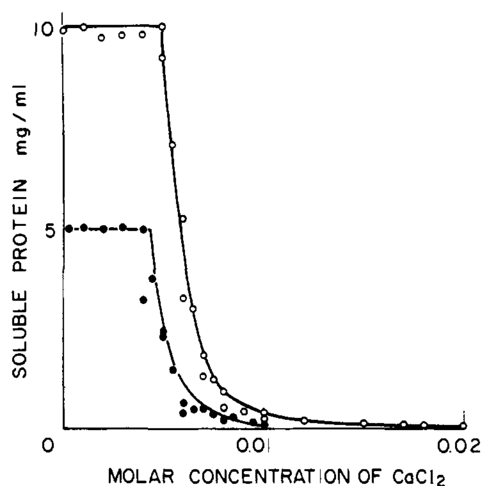

Figure 2. Solubility of calcium α_s-caseinate as a function of added $CaCl_2$ for two solutions, both in standard KCl buffer at 37°. The open circles are for a solution with an initial α_s-casein concentration of 10 mg./ml., the closed circles for a solution with an initial concentration of 5 mg./ml. The lines are calculated from eq. 1 (see text).

single aliquot addition maximal stabilization is always obtained up to calcium concentrations at the peak. As will be shown incremental addition of calcium gives different results.

For ordinate values of Figure 3 actual supernatant protein concentrations have been decremented by an amount equal to the κ-casein present initially. This is done knowing that the precipitates which form during the descending limb of and near the bottom of the dip contain less than 1% κ-casein (see below) and that the κ-casein content of prepeak precipitates is negligible compared to the total.

The striking features of Figure 3 are summarized as follows: (a) Regardless of subsequent events, the descending limbs of all curves follow closely the calcium α_s-caseinate solubility curve. Prior to precipitate formation in this region the solution becomes turbid with a characteristic gray appearance, in contrast to the white opalescence associated with micelle formation. Precipitation of calcium α_s-caseinate is progressively retarded by the presence of κ-casein; retardation being negligible at an initial ratio of 20 and obvious at an initial ratio of 10.

(b) In each case κ-casein apparently has little effect on the apparent final state of the system until the total concentration of calcium is near 0.006 M. However, at 0.007 M calcium there occurs a dramatic increase in supernatant protein with respect to calcium α_s-caseinate solubility.

(c) Between 0.01 and 0.02 M calcium, maximum micelle stabilizations are achieved. Complete stabilization in this series occurs only at an initial weight ratio of 10 and stabilization decreases as the initial weight ratio increases. Highly significant is the fact that even at an initial ratio of 20, the degree of stabilization is 4.0 to 2.0 mg./ml. over the range from 0.008 to 0.02 M calcium. For 0.5 mg./ml. of κ-casein these calcium concentrations give stabilization ratios from 8 to 4. Among the other curves maximum stabilization ratios as high as 12 are calculated. However, with an initial ratio of 12 complete stabilization is not achieved.

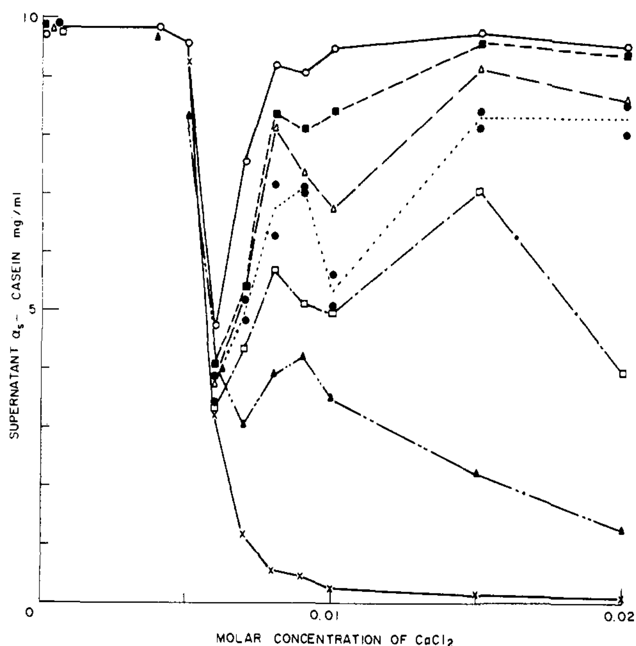

Figure 3. Supernatant protein resulting from single aliquot addition of calcium plotted as a function of $CaCl_2$ concentration for a series of α_s–κ-casein mixtures in standard KCl buffer at 37°. Each solution contained initially 10 mg./ml. of α_s-casein, but the initial κ-casein concentration varied. The different κ-casein concentrations are represented as follows: ○ for 1 mg./ml., ■ for 0.9 mg./ml., △ for 0.8 mg./ml., ● for 0.7 mg./ml., □ for 0.6 mg./ml., ▲ for 0.5 mg./ml., and × for 0.0 mg./ml. of κ-casein, *i.e.*, pure α_s-casein.

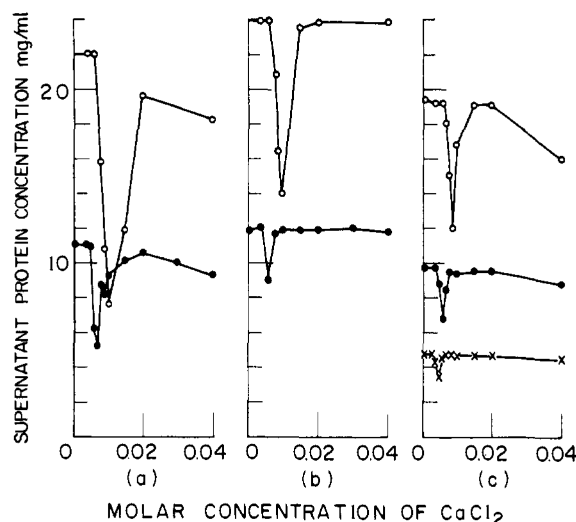

Figure 4. Supernatant protein resulting from single aliquot addition of calcium plotted as a function of $CaCl_2$ concentration for initial ratios of 10 (a), 5 (b), and for First Cycle casein (c), all at several initial protein concentrations. The symbols ○ represent data for initial α_s-casein or First Cycle casein concentrations of 20 mg./ml., ● for initial concentrations of 10 mg./ml., and × for 5 mg./ml.

Three sets of observations, which are more important to a final determination of the structure of the micelle and will be considered in detail later, are summarized briefly as follows. As the initial ratio decreases from a value of 10, complete stabilization always appears in the region of the peak. The dip, however, decreases but is still experimentally observed even at a ratio of 2.5 (Figure 1). At a ratio near 1.0 the dip may be absent. Possibly it is too difficult to observe since, as the depth of the dip decreases, the calcium concentration over which it is present becomes narrower. At constant initial ratio, as the total protein concentration increases the fraction of the α_s-casein which precipitates also increases. This situation is shown in Figure 4a and Figure 4b. Finally, as the initial ratio decreases, the slope of the descending limb of the dip becomes less negative compared to the calcium α_s-caseinate solubility curve.

Preformed micelles are unstable and give rise to characteristic precipitate formation in the region of the dip. This was shown as follows. Calcium was added to a solution containing 10 mg./ml. of α_s- and 2 mg./ml. of κ-casein to a final concentration of 0.008 *M*. Micelles but no precipitate formed at this calcium concentration. The solution was divided into three aliquots which were then diluted with 0.07 *M* KCl to final calcium concentrations of 0.004, 0.005, and 0.006 *M*, respectively. Precipitation characteristic of the dip resulted but was slow compared to the rate of precipitation when the dip is approached from the low calcium side.

The comparison given above suggests that the precipitate which forms in the region of the dip is essentially calcium α_s-caseinate. The data in Figure 3 formed the basis of an assay for small amounts of κ-casein in the presence of α_s-casein. Precipitates from the dip were harvested, brought into solution, and dialyzed against 0.07 *M* KCl. Small but varying amounts of κ-casein were added to aliquots. The stabilizing effects of these, at calcium concentrations in the region of the peak, were compared with effects on pure α_s-casein solutions treated in the same way. The details of the assay will be described elsewhere for situations where the presence of small amounts of κ-casein were in fact detected. Within the limits of the assay, which is sensitive to the presence of 1% of κ-casein, precipitates occurring in the descending limb of the dip are pure calcium α_s-caseinate.

It should be noted that the behavior described above has been found using κ-casein purified in three different ways as described under Materials and Methods. Some of these preparations, especially κ-casein purified by ultracentrifugation, have slightly lower relative stabilizing capacities indicating, as determined experimentally, that these preparations are not pure.

Single Aliquot Addition of Calcium to Unfractionated Caseins. It was considered that precipitation in the dip region might be the result of alterations in proteins produced by the preparative procedures. This possibility was examined by studying the behavior of First Cycle casein and Solubilized Skim Milk as a function of calcium concentration. The results for three First Cycle casein solutions at different initial protein concentrations are given in Figure 4c. The dip is evident in all curves, but as usual becomes more pronounced with increasing initial protein concentration. In addition, the displacement of the descending limb of the dip with concentration is observed. First Cycle casein behaves like an α_s–κ-casein solution at an initial ratio of about 7.

Solubilized Skim Milk gives a dip similar to that of mixtures of α_s- and κ-casein or First Cycle casein, except that the depth of the dip is less, probably as a result of the presence of whey protein (Figure 5).

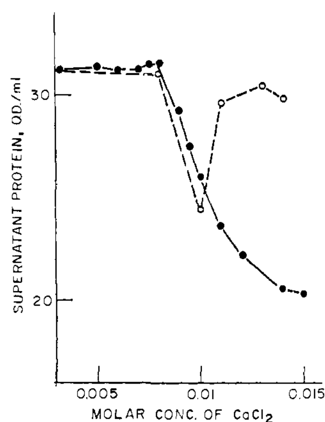

Figure 5. Supernatant protein plotted as a function of $CaCl_2$ concentration for Solubilized Skim Milk at an initial concentration of 31 mg./ml. when $CaCl_2$ is added in a single aliquot, ○, or incrementally, ●.

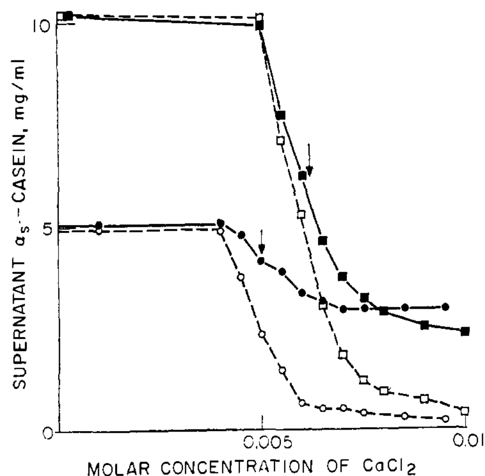

Figure 6. Supernatant α_s-casein, resulting from incremental addition of calcium, plotted as a function of $CaCl_2$ concentration for solutions containing, in standard KCl buffer, 1 mg./ml. of κ-casein and 10 mg./ml. of α_s-casein, ■; 1 mg./ml. of κ-casein and 5 mg./ml. of α_s-casein, ●; 10 mg./ml. of α_s-casein, □; and 5 mg./ml. of α_s-casein, ○. The arrows indicate calcium concentrations for the first appearance of micelles and for the bottom of the dip using single aliquot addition.

In light of these results, it seems unlikely that the characteristic behavior found for mixtures of purified caseins could be an artifact.

Incremental Addition of Calcium to α_s–κ-Casein Mixtures. Calcium was added to α_s–κ-casein mixtures in a series of increments giving final concentration changes of 0.0005 *M*. The resulting supernatant concentrations were corrected for the dilution due to calcium addition and from these values were subtracted the initial κ-casein concentrations yielding closely the concentration of α_s-casein in the supernatant. In Figure 6 is plotted supernatant α_s-casein concentration *vs.* total calcium concentration for solutions containing 10 and 5 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein (solid lines) and the corresponding calcium α_s-caseinate solubility curves (dotted lines). As observed with single aliquot addition, the calcium concentration at which precipitate first appears is nearly the same whether or not κ-casein is present. The excursion of the descending limb of the dip is also observed. The vertical arrows indicate both the location of the bottom of the dip and the first appearance of micelles on single aliquot addition. They also indicate the point where micelles first become apparent on incremental addition. Beyond the points indicated by the arrows, while the curves of single aliquot addition rise to complete stabilization, incremental addition gives increasing amounts of precipitate, extending the excursion of the descending limb but eventually leveling off well above calcium α_s-caseinate solubility at stabilization ratios between 2 and 3. The supernatant protein will increase neither with extended mixing time at a particular calcium concentration nor when the calcium concentration is extended beyond 0.01 *M*.

Again the question of the retention of native component properties during purification is raised. The effects of incremental addition have been examined with Solubilized Skim Milk. Figure 5 compares the result with single aliquot additions and reveals the characteristic differential in stabilization in the region of the peak.

The dependence of the degree of stabilization on the mode of calcium addition at once focuses attention on the question of equilibrium and suggests that stabilization may not be an equilibrium process. This matter will be dealt with in detail later.

The Effects of Ionic Strength and pH. All of the studies described thus far were carried out in standard KCl buffer. The ionic strength of this buffer was chosen after early experiments demonstrated that high stabilization ratios could be obtained under these conditions. In addition, 0.07 is the approximate ionic strength of milk.[28] If experiments with the same initial protein concentration are compared, the most noticeable effects of increasing the ionic strength are to widen the dip, making it more evident, and to lower the maximum stabilization that can be obtained at the top of the peak. In 0.3 *M* KCl, for example, stabilization ratios above 5.5 have not been obtained. As the KCl concentration is decreased below 0.07 *M*, the dip becomes narrower but the maximum stabilization ratio obtainable at the top of the peak was found not to increase significantly above 10. Complete stabilization at initial ratios appreciably above 10 has not so far been found under any conditions. Zittle and Jasewicz[29] studied the effect of NaCl concentration on the stability of casein micelles. They did not investigate calcium ion concentrations below 0.01 and did not detect the dip. However, they did find stabilization weight ratios as great as 10 but no greater. Stabilization ratios of 10 have also been reported by Zittle,[30] Zittle, *et al.*,[31] and Pepper and Thompson.[32]

Variation in pH from 6 to 7.5 has little or no effect on the stabilization behavior of α_s–κ-casein mixtures. When calcium adjusted to pH 7.2 is added to an α_s–κ-casein mixture at the same pH the pH drops, indicating that calcium ions displace hydrogen ions on the protein molecule. When calcium is added to a solution con-

(28) "Handbook of Biological Data," William S. Spector, Ed., W. B. Saunders Co., Philadelphia, Pa., 1956, p. 50.
(29) C. A. Zittle and L. B. Jasewicz, *J. Dairy Sci.*, **45**, 703 (1962).
(30) C. A. Zittle, *ibid.*, **44**, 2101 (1961).
(31) C. A. Zittle, M. P. Thompson, J. H. Custer, and J. Cerbulis, *ibid.*, **45**, 807 (1962).
(32) L. Pepper and M. P. Thompson, *ibid.*, **46**, 764 (1963).

taining 10 mg./ml. of α_s-casein and 1 mg./ml. of κ-casein to give a final concentration of 0.12 M the pH drops from 7.2 to 6.7. If the protein solution is buffered at pH 7.2 with 0.01 M imidazole the pH drops to 7.05. Experiments were performed in which the initial pH values of the protein solutions were varied from 7.5 to 6.5, and some solutions were buffered with 0.01 M imidazole and others were not. Within experimental error, variations in pH and buffering had little or no effect on the stabilization behavior of the solutions.

The Degree of Association of α_s-Casein and κ-Casein in the Absence of Calcium. The studies just presented giving the effects of calcium on supernatant protein question the relevancy of an α_s–κ-complex of the type observed by Waugh and von Hippel to the formation of casein micelles. These matters will be discussed below. The work reported in this section was undertaken to aid in establishing the extent to which complexes may form in the absence of calcium and to establish some of their characteristics.

Complexes were initially sought on the assumption that a maximum stoichiometry limits the stabilizing capacity of κ-casein. Light scattering studies were first used. The rationale was to prepare a protein solution with the assumed maximum stoichiometric ratio by adding calcium incrementally to a solution containing an excess of α_s-casein, thereby precipitating excess α_s-casein and harvesting the stoichiometric ratio as micelles in solution. After removal of calcium, light-scattering studies would then reveal the size of the complex or complex aggregates. A study of size *vs.* pH would then be expected to reveal a plateau, corresponding to the unit complex, lying between aggregates of complexes and their constituent monomers.

A reproducible stoichiometric ratio was not found due to the fact that the dip was entered to varying extents. When solutions containing initial ratios of 3.5 and 4 were examined τ/Hc was found to decrease steadily with increasing pH from 1.2×10^6 at pH 7 to near 30,000 at pH 12, approximately the monomer size of the individual caseins. The results showed a great deal of scatter.

In another attempt to demonstrate complexing, an α_s-casein solution, a κ-casein solution, and an α_s–κ-casein mixture of initial ratio 4 were examined by starch gel electrophoresis at room temperature using a Poulik tris–citrate gel containing no urea. The α_s–κ-casein mixture showed no new bands and there was no change in the mobility of either of the component bands.

Casein solutions were examined extensively by analytical ultracentrifugation and it was by this technique that interaction was demonstrated, although at a weight ratio much less than 4 and not at low temperature.

Many experiments were carried out in the ultracentrifuge at 2–6° using several initial ratios between 4.5 and 1 and the buffers given under Methods. The resulting patterns showed characteristic α_s- and κ-casein peaks. Attempts were made to analyze these for systematic alterations in relative area with the result that there was no indication of interaction. Pretreatment with urea or high pH did not alter these patterns. This situation is similar to that at 20° without pretreatment.

In Figure 7 are shown schlieren patterns obtained when casein solutions in pH 7 phosphate buffer, with an ionic strength of 0.1, were centrifuged at 20° at a speed of 59,780 r.p.m. The first row gives typical patterns for a 2-mg./ml. solution of κ-casein, the second and third rows give patterns for a solution containing 2 mg./ml. of κ-casein and 9 mg./ml. of α_s-casein, and the fourth row gives patterns for a solution containing 9 mg./ml. of α_s-casein. The patterns for the α_s–κ-casein mixture are seen to be the sum of the patterns for the other two solutions. The sedimentation coefficient of the peak of the α_s-casein solution is 3.9 S.; that for the slow peak of the α_s–κ-casein mixture is 3.9 S. The κ-casein peak and the fast peak of the mixture are too polydisperse to permit accurate determination of the sedimentation coefficients, but they are both near 15 S. There is no indication of interaction between α_s- and κ-caseins.

In Figure 8 are shown schlieren patterns obtained when casein solutions were centrifuged at 37°, pH 7, 0.07 M KCl, and 59,780 r.p.m. The first row shows two patterns for 2 mg./ml. of κ-casein, the second, patterns for a mixture containing 2 mg./ml. of κ- and 7 mg./ml. of α_s-casein, and the last, patterns for a solution containing 7 mg./ml. of α_s-casein. Here the pattern for the mixture is not the sum of the patterns for the other solutions. The sedimentation coefficients of the α_s-casein solution and of the slow peak of the mixture are identical, s_{20} = 3.4 S. The sedimentation coefficient of the κ-casein peak is s_{20} = 18.8 S. The patterns for the mixture show no 18.8 S. peak; however, there is a new peak with s_{20} = 7.0 S. This is believed to be due to an α_s–κ-casein interaction product. In addition, the slow peak of the mixture is slightly smaller than the peak of the α_s-casein solution, which may be used to indicate the amount of α_s-casein involved in the 7.0 S. peak. The α_s-casein to κ-casein weight ratio in the 7.0 S. peak must of course be much less than 3.5, and it appears to be approximately unity. This product dissociates on cooling to give patterns of the type shown in Figure 7. In fact, patterns of this type are obtained when calcium is removed from an appropriate micelle system at 37° and the resulting protein solution is cooled to 2°.

To test further for interaction, a sedimentation experiment was carried out at 37° on a solution containing 5 mg./ml. each of α_s- and κ-caseins. A single peak was observed with a sedimentation coefficient of 8.0 S.

Patterns also were obtained on casein solutions that had been treated with 4 M urea prior to dialysis *vs.* ionic strength 0.1 phosphate buffer at pH 7. These were examined at a temperature of 20°. Patterns similar to those of Figure 8 and not like those of Figure 7 were obtained. Apparently pretreatment with urea induces a low ratio α_s–κ-casein interaction product at 20°.

Further evidence showing that the peaks of the α_s–κ-mixtures, arranged by increasing s_{20}, probably represent α_s-casein, an α_s–κ-casein interaction product, and κ-casein, was shown by centrifugation in the SW 39 swing bucket rotor of the preparative ultracentrifuge. These experiments were performed under conditions

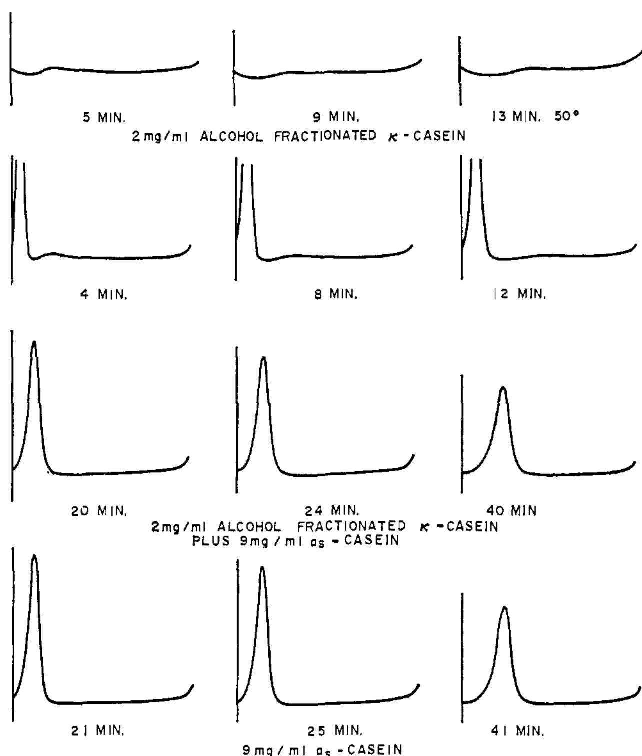

Figure 7. Sedimentation schlieren patterns obtained by centrifuging casein solutions at 20° in 0.1 ionic strength phosphate buffer at pH 7 and a speed of 59,780 r.p.m. The angle of the phase plate is 60° unless otherwise specified. For details see text.

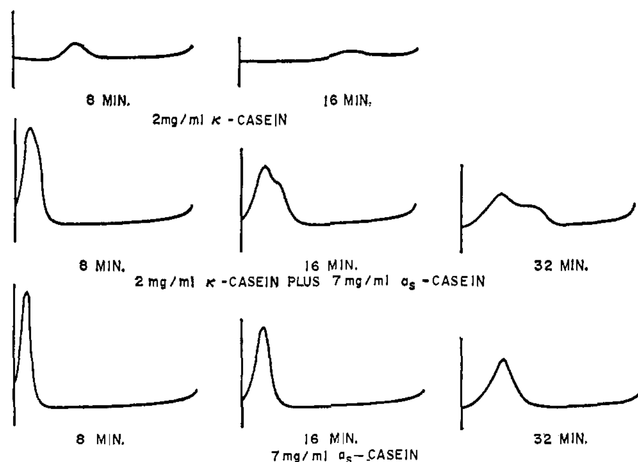

Figure 8. Sedimentation schlieren patterns obtained by centrifuging casein solutions at 37°, 0.07 M in KCl at pH 7.2 and at a speed of 59,780 r.p.m. The angle of the phase plate is 60° in all cases. For details see text.

where complexing was not expected (low temperature) and where it was (high temperature).

For conditions where complexing is not expected centrifugation of two 5-ml. samples was carried out at 39,460 r.p.m. for 7 hr. at 4°; one sample contained 2 mg./ml. of κ-casein and 7 mg./ml. of α_s-casein, and the other 3 O.D. per ml. of fraction S plus 7 mg./ml. of α_s-casein. Each tube was sectioned into seven portions including a pellet which was dispersed in distilled water. Calcium was added to each fraction at 37° to final concentration of 0.03 M. In fractions and 1 and 2 (centripetal) of each tube precipitates formed in amounts indicating an initial ratio of at least 15. The dispersed pellet, upon calcium addition, first became opalescent and then developed micelles over a period of several hours, a result characteristic of ratios near unity.

For conditions where complexing is expected, centrifugation was carried out at 37° on three 5-ml. samples of a solution containing 2 mg./ml. of κ-casein and 7 mg./ml. of α_s-casein. The centrifugation conditions were 39,000 r.p.m. for 4.75 hr. Portions collected and combined were the upper 1.5 ml., the next 2.5 ml., and the remaining solution plus pellet. Each portion was then divided into three aliquots and calcium was added to give final concentrations of 0.008, 0.02, and 0.2 M. Aliquots of the original uncentrifuged solution gave no precipitate. Precipitate formed only in portions 1 and 2 and only at 0.2 M calcium, where in each case it amounted to ~35% of the protein. Therefore, the initial ratio in portions 1 and 2 had been increased by centrifugation to approximately 7. Thus κ-casein had moved preferentially to the base of the tube. No precipitate formed in any of the other tubes.

The results presented, which clearly differ from those obtained by Waugh and von Hippel,[5] are based on preparative procedures which use citrate to sequester calcium. In order to gain further information, preparations were made according to the earlier oxalate procedure used to obtain First Cycle casein, fraction S, and fraction P. At 2°, mixtures of fraction S and fraction P gave patterns having peaks corresponding to those of fraction S and fraction P individually, including those corresponding to α_s- and β-caseins at ~5 and 3 S., respectively, and a κ-casein peak, at $s_{20} \sim 15$ S., with its characteristic rapid boundary spreading. However, the patterns obtained using First Cycle casein (oxalate) or First Cycle casein (citrate) are equal to each other but differ from all patterns described here or elsewhere. In each, two peaks of approximately 3 and 5 S. are present but there is no characteristic κ-peak. Instead there is a shoulder on the 5 S. peak which disappears rapidly without giving rise to an independent boundary.

One further attempt was made to find a rational explanation for the variety of results obtained. This was to add up to 0.003 M calcium to a mixture containing, per ml., 8 mg. of α_s-casein and 2 mg. of κ-casein. Surprisingly, the pattern obtained at 2° was identical with that obtained in the absence of calcium (Figure 7).

Calcium Ion Binding and α_s-Casein Solubility. If there were no calcium binding to α_s-casein the solubility curves would be independent of protein concentration. Displacement along the abcissa with increasing protein concentration either of the calcium concentration associated with initial precipitation or the total calcium concentration to give particular levels of protein solubility may be used to calculate calcium binding. The first displacement is related to the calcium bound to the protein just prior to precipitation plus the mass action effect of the protein, and the second displacement is a measure of the calcium bound to the protein in the precipitate. If the protein mass action effect is neglected the first approach and the data of Figures 2 and 6 lead to 8 or 6 calcium ions bound per $\alpha_{s1,2}$-casein molecule of mol. wt. 27,300. The second approach and the data of the same figures give, between 5 and 2 mg./ml. of soluble protein, an average value of 11 calcium ions bound per molecule. It is noted that the α_s-casein

molecule contains 9 organic phosphorus atoms per molecule[15] and that infrared studies of calcium binding by Ho and Waugh[33] show that organic phosphate groups are the primary but not sole binding sites.

The solubility product for the formation of calcium α_s-caseinate precipitate should ideally have the form of eq. 1

$$K = [\alpha\text{-Ca}_m][\text{Ca}]^n \quad (1)$$

where K is an equilibrium constant, α-Ca$_m$ is the solution calcium α_s-caseinate which engages in precipitate formation, and n is the additional calcium interaction required to form the precipitate.[34] Both α-Ca$_m$ and n are involved in determining the calcium concentration for initial precipitation, while n alone determines the shape of the curve. An exponent giving a reasonable fit for shape is 4, values of 3 and 5 being acceptable, but deviating more than 4. In Figure 6 the dotted lines correspond to eq. 1, using $n = 4$ and $m = 7$. The value of K is then 1.3×10^{-13} and the corresponding ΔF is -4.4 kcal./mole of calcium ion bound.

We noted above that the precipitate contains 11 calcium ions per α_s-casein molecule. The species in solution then should have 7 calcium ions bound, in reasonable agreement with experimental values. If calcium ions are accepted individually, a general equilibrium constant near 10^{-6} (ΔF about -8.3 kcal./mole) is sufficient to allow the protein to remain in solution just prior to the rapid production of precipitate.

Stabilization at Low Temperatures. The ultracentrifuge patterns described above indicate that α_s- and κ-caseins do not mutually interact in the absence of calcium at 2°. Attempts were made to form micelles at 2°, using a system containing initially 4 mg./ml. of α_s- and 1 mg./ml. of κ-casein. In all cases supernatants after assay centrifugation were clear. The supernatant protein concentration minus the initial κ-casein concentration levels off at values near unity, the trend being to decrease as the calcium concentration increases. Comparison of these results with the solubility of calcium α_s-caseinate under the same conditions indicates stabilization at a weight ratio of $\sim$0.5.

Described above is a concentration dependence in which increasing concentration reveals, for example, an increasing dip width and depth. The ability of κ-casein to stabilize α_s-casein at low temperature is shown by the fact that a solution containing 15 mg. each of κ- and α_s-caseins at 0° becomes no more than opalescent in the presence of 0.018 M calcium. At 0.2 M calcium, calcium α_s-caseinate precipitate forms at 0° to the extent of 5 mg./ml. and the supernatant (stabilizing ratio 0.85) again is no more than opalescent. In both cases raising the temperature of the supernatants to 37° increases turbidity without precipitate formation. Ultracentrifugation at 6° of a mixture containing 5 mg./ml. each of α_s- and κ-caseins at 0.02 M calcium was performed. At 10,000 r.p.m. a small amount of colloid was rapidly removed. After accelerating to 59,780 r.p.m., the pattern consisted of a single peak of $\sim$17 S. which spread rapidly but preferentially toward lower values of s. A small amount of low molecular weight material initiated a peak which remained near the meniscus.

(33) C. Ho and D. F. Waugh, *J. Am. Chem. Soc.*, 87, 889 (1965).

(34) The situation is probably not this simple. Ultracentrifuge patterns at 37° of solutions containing α_s-casein plus calcium reveal, between 0.001 and 0.002 M calcium, a single peak with a definite fast shoulder which progressively moves into an ill-defined second peak. At 0.004 M calcium the pattern reveals an increased polydispersity with a shift toward faster sedimenting components but with slowly sedimenting materials still present.

Discussion

The extent of precipitation of calcium α_s-caseinate after the addition of small amounts of calcium to α_s–κ-mixtures having initial ratios as low as 2.5 shows that most of the α_s-casein is free in solution just prior to micelle formation. In the region of the descending limb of the dip at 37° only a small divergence is seen between the calcium α_s-caseinate solubility curves and supernatant protein curves of mixtures (see Figures 3 and 6). Further, the calcium concentrations at which precipitates first appear are the same within experimental error for pure α_s-casein solutions and mixtures having the same α_s-casein concentrations. Even if the system consisted initially of high ratio interaction products, these must dissociate to a major extent to give the descending limb of the dip. The possibility that low ratio interaction products exist just prior to micelle formation will be considered below.

It is obvious that the micelle cannot be constructed completely of pre-existing complexes. It is also apparent that the rapid increase in stabilization which accompanies micelle formation in the ascending limb of the peak involves calcium; thus, that calcium and κ-casein are both required for micelle formation and for α_s-casein stabilization.

Waugh and von Hippel[5] and Waugh[6,7] have suggested that micelles are formed by the linking together primarily of pre-existing complexes having a weight ratio of 4. It was suggested also that nonstoichiometric complexes containing less α-casein could be involved and that they decreased micelle size. A scheme for the complex which helped to account for certain clotting properties of the system was proposed. The maximum stoichiometry was expected to limit the stabilizing capacity of κ-casein. It is clear that this scheme cannot account either for the occurrence of the dip or for observed stabilization ratios well in excess of 4.

The incremental addition of calcium always gives less stabilization than single aliquot addition up to the calcium range of the peak. In the region of the peak the difference is striking: stabilization ratios of 10 to 12 as compared with 2. This dependence on path of the degree of stabilization is interpreted to mean that this is a nonequilibrium system under our experimental conditions; the equilibrium state thus cannot be defined. The nonequilibrium nature of the system and the implications of this with respect to micelle structure will be explored in part II of this series.

Are there important interactions of α_s- and κ-caseins prior to micelle formation? In the absence of calcium and at a time when only impure fractions were available, Waugh and von Hippel[5] obtained considerable evidence for the existence of a complex of α_s- and κ-caseins variable in composition but having a preferred maximum stoichiometry corresponding to a weight ratio of 4 (α_s/κ). This ratio coincided with the abundances of α_s- and κ-caseins found in milk. The preferred complex, which was observed necessarily at low temperature (2°) to avoid interaction with β-casein, had $s \sim 7.5$ S. The latter sedimentation coefficient

was sensitive to high pH treatment. The spontaneous occurrence of a complex of s_{20} = 7.5 S., associated with a skewed peak, has been reported by Pepper and Thompson[32] and observed even for dephosphorylated proteins. Treatment at high pH, however, did not alter the s_{20} of the complex they obtained. Swaisgood and Brunner[22] find that spontaneous complexing does not occur at room temperature but only after pretreatment with urea or high pH when complexing takes place at a ratio of 4. The sedimentation coefficients of the complexes obtained by these two treatments differ markedly: 6.2 S. for the alkaline-treated and 4.7 S. for the urea-treated materials. Garnier, *et al.*,[35] report spontaneous complexing at 25° only at a ratio of unity to give a product of s_{20} = 14.7 S., and report further that the liberation of protons on rennin action (over a wide range of temperature) also supports the existence of an α_s–κ-casein interaction product of weight ratio near unity. All experiments were carried out in 0.1 *M* NaCl at pH 6.95.

Our present data suggest that interaction occurs spontaneously at 37°, requires pretreatment with urea or high pH in order to occur at 20°, and occurs neither spontaneously nor after such pretreatment at temperatures near 5°. When interaction occurs the interactant has a weight ratio near unity and a sedimentation coefficient near 7.5. Obviously the present patterns obtained with First Cycle casein represent a new type not previously reported.

When we add the observations that peaks are usually skewed and boundary spreading is most often greater than anticipated, it must be concluded that, in the absence of calcium, α_s–κ-casein interaction products in these complex systems, the simplest of which start out as individual α_s- and κ-casein polymers, are not monodisperse and are probably not of constant composition. Apparently a wide variety of interaction patterns is possible. A particular pattern seems to be dependent on the components present and may depend in unknown ways on the type of milk and the preparative procedure but is, apparently, temporarily reproducible.

There is a complication which, if an analysis were to be carried further, must be considered. It is that these are multicomponent interacting systems for which a theory of sedimentation analysis has not yet been derived. If re-equilibration occurs during centrifugation, one cannot expect a one symmetrical peak–one component relationship, but something far more intricate.[36]

Whatever the structure of the system in the absence of calcium, the only interaction product that can survive during the descending limb of the dip is one having a low weight ratio. The existence of a low weight ratio interaction product in the presence of calcium prior to micelle formation is consistent with results reported here and in fact certain observations can be accounted for readily on this basis. For example, there is a progressive divergence of curves for calcium α_s-caseinate solubility and descending limbs for α_s–κ-casein mixtures as shown, for example, in Figure 6. Just prior to the calcium concentration where micelles start forming, 1 mg. of κ-casein has stabilized about 1–1.3 mg. of α_s-casein. Beyond the point of micelle formation the curves diverge further until 1 mg. of κ-casein can stabilize approximately 2 to 2.5 mg. of α_s-casein. The latter values must, of course, include some micelles whose α_s-casein content might be, and probably is, higher than the low weight ratio interactant under consideration. This statement assumes that all κ-casein is utilized and is not bound significantly in the precipitate.

Equation 1 can be used to calculate the expected origins and shapes of supernatant protein curves for systems having present low weight ratio α_s–κ-casein interaction products. If the initial ratio is above approximately 4, one can conclude that a unit weight ratio interaction product in which normal calcium binding is preserved will not produce significant displacement of the calcium concentration at which precipitation is initiated. For lower initial ratios, the descending limb of the dip becomes difficult to observe experimentally and to define quantitatively. At initial ratios higher than about 4, a solubility differential related to the extent of interaction is expected and is observed as described above. To calculate this solubility differential, eq. 1 is probably not applicable[34] and has not been used.

Another result appears to be consistent with the presence of low weight ratio interactants. This is the fact that the dip decrements as the weight ratio decreases and is small but apparent at an initial ratio of 2.5. The dip would be expected to disappear when the initial ratio equals the weight ratio, or average weight ratio, of the interaction product(s). Techniques have not been refined to the point where this expectation can be tested. In this respect it is noted that the entire dip at an initial ratio of 2.5 covers a calcium range of 0.0003 *M* and has a depth of about 10% of the protein content (Figure 1).

The assumption of some type of specific interaction of α_s- and κ-caseins in the formation of casein micelles is mandatory, as is evident from the necessary and sufficient conditions for micelle formation and for the subsequent incorporation of β-casein. We conclude that the hierarchy of interactions which are necessary for micelle formation *may* start in the absence of calcium with an α_s–κ-casein interaction product having a weight ratio near unity. Calcium is necessary to utilize this interaction product, or a similar product formed in the presence of calcium, in the first important interaction required for the formation of micelles and for the stabilization of calcium α_s-caseinate.

Specificity of interaction does not in itself eventually lead to the formation of α_s–κ-casein complexes in which interaction sites on κ-casein dictate a maximum stoichiometry and by this mechanism a stabilization ratio. If a maximum stoichiometry leading to a weight ratio of 10 is assumed, the data of Figure 3 cannot be readily accounted for, particularly the stabilization behavior at initial ratios above 10. The situation is more complex than simple stoichiometry can accommodate. These complications will be considered in part II of this series.

At calcium concentrations where micelles first make their appearance, our data taken together with the infrared data of Ho and Waugh[33] suggest that calcium has interacted essentially with all of the organic phosphate groups present in the system. It is clearly indicated that interaction with a protein group other than phosphate may in fact be involved in the micelle-forming reaction.

(35) J. Garnier, J. Yon, and G. Mocquot, *Biochim. Biophys. Acta*, 82, 481 (1964).

(36) H. Fujita, "Mathematical Theory of Sedimentation Analysis," Academic Press Inc., New York, N. Y., 1962, p. 129.