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k-Casein and the Stabilization of Casein Micelles

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The casein micelle contains as calcium caseinates β-casein and a complex of α-casein and k-casein. k-Casein is the most important single factor responsible for micelle stabilization and is the protein on which rennin acts immediately. The relative abundances are about 55% α-casein, 30% β-casein and 15% k-casein. A solution containing essentially β-casein and the α-k-complex may be prepared by removing calcium from micelles (first cycle casein). If first cycle casein is treated with 0.25 M $CaCl_2$ at 37° and pH 7, the α-k-complex is split, β-casein and α-casein rapidly form a precipitate, which will yield second cycle casein, and k-casein forms polymers of ~13.5 S which remain in the supernatant. These k-casein polymers are insensitive to calcium ion concentration and temperature but dissociate (reversibly) when brought to pH 12 to form monomers of ~1.3 S, similar to α- and β-casein monomers. k-Casein has an electrophoretic mobility close to that of α-casein but contains about one-half as much phosphorus as α-casein and in the presence of rennin undergoes a rapid, time dependent precipitation. Second cycle casein and supernatant containing k-casein have been recombined. In the weight ratio of 4α-/$1k$-, the α-k- complex re-forms spontaneously at pH 7, before or after being titrated to pH 12, and stable micelles form on adding calcium chloride. With higher ratios, excess α-casein precipitates on adding calcium chloride, and with lower ratios the excess k-casein forms independent polymers. Titration to pH 12 converts acid precipitated casein into essentially first cycle casein. The "α-casein" obtained from acid precipitates, which in the past has received considerable attention, thus is derived from the α-k-complex and contains both proteins. It is suggested that k-casein exerts its primary stabilization in the formation of the α-k-complex, which process reduces the number of calcium sensitive groups of α-casein so situated that calcium may act as an interpolymer or intercomplex link. β-Casein may also be important in stabilization of micelles since first it enters the series of polymerization reactions which are, among other variables, sensitive to temperature and to calcium ion concentration and second, β-casein does not precipitate in the presence of calcium chloride but forms a colloidal suspension.

In a previous publication,[2] a method for preparing caseins at constant pH has been described, the preparation being a mixture of α- and β-casein approximately in the proportion in which they occur in skim milk. The procedure, based on observations that the constituents of the casein micelle are in equilibrium with similar constituents free in solution,[3] isolates α- and β-casein from other materials by application of a cycle, illustrated in Fig. 1 and consisting of the steps: (1) shifting of essentially all casein into the micellar form by the addition of calcium ion and centrifugation of micelles from solution; (2) removal of calcium from micelles (thereby releasing casein) by adding potassium oxalate and oxalic acid, removal of calcium oxalate by centrifugation and removal of excess potassium oxalate by dialysis. The product has been referred to as soluble casein. The material obtained by applying two such purification cycles has been partially characterized by physical studies.[2]

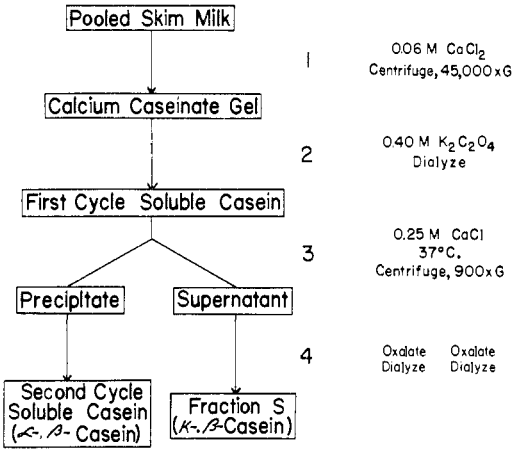

Fig. 1.—Schematic flow sheet outlining the procedure used to prepare first cycle soluble casein, second cycle soluble casein and fraction S.

The casein micelle as found in milk has properties which make it of unusual interest. For example, as previously stated it is in equilibrium with its constituents free in solution, the size distribution of micelles is relatively narrow,[4,5] micelles do not readily heat coagulate and yet, on the addition of rennin, suspensions of these relatively stable colloidal particles are transformed into gels. Interest in the underlying physical and chemical factors responsible for the behavior pattern of micelles suggested that soluble casein be examined with respect to its capacity to reconstitute, on the addition of calcium, micelles similar to those found in skim milk.

The product vsed in physical studies[2] was obtained after the application of two of the cycles outlined above (second cycle casein). It was found that the addition of calcium to this material at concentrations markedly lower than those found in skim milk led invariably to the formation of a coarse, heavy precipitate which in no way resembled casein micelles. At the same time it was known[3] that the addition of calcium to soluble casein obtained after the application of a single cycle (first cycle casein) led to the formation of stable micelles which closely resembled in their properties the micelles of skim milk. First and second cycle caseins did not, however, differ in any immediately obvious way, a circumstance which was found to result from the manner in which the stabilizing factor to be described is incorporated with α-casein in the casein micelle.

It was clear that if a stabilizing factor were involved, this factor was being removed at the start of the second purification cycle, presumably dur-

(1) Predoctoral Fellow of the National Science Foundation, 1952–1955. Post-doctoral Fellow of the National Heart Institute (National Institutes of Health), 1955–1956. A part of this work was reported in P. H. von Hippel, Ph.D. Thesis, Department of Biology, Massachusetts Institute of Technology, 1955.

(2) P. H. von Hippel and D. F. Waugh, THIS JOURNAL, **77**, 4311 (1955).

(3) D. F. Waugh, L. Varga and R. Scuderi, unpublished.

(4) H. Nitschmann, *Helv. Chim. Acta*, **32**, 1258 (1949).

(5) H. Hostettler and K. Imhof, *Milchwissenschaft*, **6**, 351, 400 (1951).

ing the addition of calcium which results in the formation and precipitation of insoluble calcium caseinate from first cycle casein. In addition, the factor was clearly non-dialyzable, otherwise it would have disappeared during the removal of the excess oxalate at the end of the first cycle. The supernatant obtained after calcium addition and centrifugation, at the start of the second cycle, was freed of calcium ions by oxalate titration and was then combined with second cycle casein in various proportions. An appropriate mixture of supernatant and second cycle casein, on the addition of calcium in amounts similar to those found in skim milk, yielded stable micelles which clotted on the addition of rennin. The factor responsible for micelle stabilization has been found to be a member of the casein complex. We will refer to it as *k*-casein. At this time we consider the preparation and certain of the properties of *k*-casein. A subsequent publication will consider *k*-casein as the primary target of rennin.[6]

Materials and Methods

Purification and Fractionation of the Casein Complex.—The preparation of second cycle soluble casein is illustrated schematically in Fig. 1 and was carried out as described,[2] except that after adding the calcium chloride at the start of the second preparative cycle, the solution was heated to approximately 37°. This favors the formation of larger calcium caseinate particles and thus makes it easier to separate them from the supernatant. This modification also improved the yield of second cycle casein.

The supernatant remaining after the removal of the calcium caseinate precipitate at the beginning of the second preparative cycle was treated as follows.

A. Removal of all Insoluble Calcium Caseinate.—The supernatant (~300 ml. arising from 1 liter of skim milk) was centrifuged for 120 minutes at 5° and 90,000 × *g* (Spinco Preparative Ultracentrifuge, Model L, Rotor #30, 28,000 r.p.m.). The precipitate was discarded.

B. Removal of Calcium.—1. Calcium was removed from the supernatant by titrating with 80 ml. of 1.5 *M* potassium oxalate at 0° and constant *p*H. The *p*H was measured continuously and maintained at *p*H 6.6–7.0 by the addition of 0.1 *M* oxalic acid, as needed.

2. Insoluble calcium oxalate, present in a finely divided state, was removed in two stages: an initial centrifugation for 30 minutes at 5° and 895 × *g* (International Refrigerated Centrifuge, bucket type rotor, 2,000 r.p.m.) followed by a centrifugation for 90 minutes at 5° and 90,000 × *g* (Spinco Preparative Ultracentrifuge, rotor #30, 28,000 r.p.m.).

3. The excess potassium oxalate was removed by dialysis against two six-liter lots of 0.15 *M* KCl.

C. Storage of Product (Fraction S).—The dialyzed solution was shell-frozen in large Pyrex tubes, using liquid nitrogen, and stored at −20°. It was stable for many months in this condition. This product is designated Fraction S in the subsequent discussion.

Measurement of Protein Concentrations.—The quantitative biuret technique used has been described.[2]

Reagents and Buffers.—Analytical reagent grade chemicals were used throughout.

The phosphate buffer used in electrophoresis and ultracentrifugation experiments carried out at neutral *p*H contains per liter: 6.97 g. of K_2HPO_4, 3.63 g. of KH_2PO_4 and 3.95 g. of KCl. This buffer has a *p*H of 6.98 and an ionic strength, ($\Gamma/2$), of 0.20.

The physico-chemical experiments performed at elevated *p*H were carried out using a phosphate buffer containing per liter: 8.71 g. of K_2HPO_4 and 3.22 g. of KOH. This buffer has a *p*H of 12.3 at 20° (12.4 at 5°) and a $\Gamma/2$ of 0.19. When a 1% casein solution was made up in this buffer, the *p*H dropped to approximately 12.1 at 5°.

(6) P. H. von Hippel and D. F. Waugh. Studies have been made of the action of rennin on first cycle casein, second cycle casein, α-, β- and *k*-caseins separately and in mixtures, and micelles reconstituted from these proteins. These studies will be reported in detail.

Ultracentrifugation.—Details of ultracentrifugation[2] and of the measurement and control of rotor temperature[7] have been described.

Electrophoresis was carried out as described[2] and in the Model H Unit built by the Specialized Instruments Corporation, Belmont, California.

Rapid-mixing Technique.—Qualitative experiments designed to investigate the effect of ionic calcium and rennin on the various casein components were carried out as follows. Aliquots of 2 ml. of protein solution were placed in 3-ml. test-tubes and suspended in a rack in a 37° temperature bath. Additional reagents (*e.g.*, $CaCl_2$) were added to these solutions using a 1-ml. syringe equipped with a hypodermic needle to the end of which was attached a circular nylon disk having a diameter just slightly smaller than the internal diameter of the test-tubes used. This device made it possible to add small amounts of various reagents to the thermostated protein solutions and simultaneously to mix them, the reagents being expressed from the syringe as the nylon disk descends through the solution.

Results

From our previous examination of preparations of soluble casein containing α- and β-casein,[2] it is clear that, in the absence of calcium, these proteins exhibit a series of polymerizations which are dependent primarily upon *p*H and temperature[2] and on ionic strength.[1] At *p*H 7 and ~4°, most of the β-casein is presented as monomers and α-casein (and possibly some β-casein) as polymers of $S_{20} \sim 4.5$ *S*. On increasing the temperature there is a gradual incorporation of β-casein into polymers while, at the same time, the mean sedimentation coefficient of the polymers markedly increases. Increasing the *p*H at 4° causes a decrease in the average size of the α-casein polymers present until, at *p*H ~ 10.8, the α-casein polymers dissociate and the solution contains only α- and β-casein monomers with an average $S^\circ_{20} = 1.18$ *S* and $D^\circ_{20} = 7.11 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. The molecular weight of α-casein probably lies in the range of 13,000 to 15,000 and that of β-casein in the range of 15,000 to 25,000. An average axial ratio of 12 was calculated for the monomers in the mixture.[8]

An examination of fraction S suggested that *k*-casein also polymerizes and depolymerizes, although the behavior of *k*-casein is different from that of either α- or β-casein. Details are given below.

The Effect of Ionic Calcium on α- and β-Casein.—Addition of ionic calcium to second cycle casein at 0° to give a final concentration of 0.07 *M* produces a silty precipitate consisting mainly of α-casein, the β-casein remaining in solution in monomeric form. The clear supernatant, on warming to 37°, becomes opaque, since the calcium β-caseinate forms a colloidal suspension. The supernatant obtained after removing this colloid is essentially free of protein. Addition of ionic calcium to second cycle casein at 37° leads to precipitation of both α- and β-caseins.

Thus, calcium α-caseinate is insoluble throughout

(7) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Instr.*, **23**, 609 (1952).

(8) The method used in ref. 2 to calculate axial ratios from values of *S* and *D* is incorrect. Perrin's equation as used leads to axial ratios of hydrated molecules and not anhydrous molecules as stated. On recalculation, a considerably higher value of kinetic water is required to reconcile viscosity and sedimentation and diffusion data. This amount of water, 0.8 to 1.0 g. water per g. protein, results in axial ratios of 8.1 and 7.1 (hydrated) and 11 and 10 (anhydrous). These values and the average casein molecular weight (15,000) lead to particles having an anhydrous major axis of 162 or 153 Å. and anhydrous minor axes of 14.8 or 15.2 Å.

the temperature range used, while β-casein, in agreement with the observations of others,[9] shifts progressively into the form of colloidal aggregates as the temperature is raised. Experiments with α- and β-caseins fractionated by calcium precipitation[10] substantiated the solubility findings given above.

Physical Examination of Fraction S in the Absence and Presence of Calcium.—The supernatant obtained from the first step of the second purification cycle (fraction S) was examined in the ultracentrifuge and electrophoresis apparatus at *p*H 7 and *p*H 12.

In the ultracentrifuge at *p*H 7, fraction S exhibits two peaks, one sedimenting with $S_{20} = 13.3$ *S* and the other with $S_{20} = 1.3$ *S*. Approximately 70% of the total protein sediments with the fast peak, which, though it appears normal, reveals abnormally rapid boundary spreading and is thus probably somewhat polydisperse. Sedimentation patterns were obtained using fraction S at a total protein concentration of 0.4% in *p*H 6.98 phosphate buffer and at temperatures ranging from 1.0 to 31.65°. At all temperatures in this range, the relative areas and sedimentation coefficients of the two peaks were found not to be significantly changed.

We investigated the effect of high *p*H by diluting a fraction S preparation with *p*H 12.4 buffer to a total protein concentration of 0.37%. At 1.1° a single, apparently monodisperse component sedimenting at $S_{20} = 1.31$ *S* was observed. At 23.3° an identical pattern was seen, the sedimentation coefficient of the single component being 1.27 *S*.

The dissociation into monomers at high *p*H appears to be entirely reversible between 0 and 24°, the temperatures studied, just as it is for α-casein. Fraction S was titrated to *p*H 12 with 0.15 *M* KOH at 0°, held at this *p*H for 2.5 hr., and then returned to neutrality by dialyzing against *p*H 7 buffer. The resulting solution was examined in the ultracentrifuge, and the *p*H 7 pattern was seen to be entirely restored.

Electrophoretic runs were made in phosphate buffer at *p*H 7, where polymers are present and at *p*H 12, where complete dissociation into monomers has taken place. In the patterns obtained at both *p*H values, a large and a small (~30%) peak were observed. The small peak migrated in each instance with a mobility close to that of β-casein, while the fast peak had, at *p*H 7 and *p*H 12, mobilities within 1 cm.^{2} volt^{-1} sec.$^{-1}$ of those of α-casein under the same conditions.[2] Pure fractions are currently being examined.

Ionic calcium appears to have no effect on fraction S. When $CaCl_2$ is added to fraction S to give final calcium concentrations up to 1 *M* at either 0 or 37°, the solution remains perfectly clear. The pattern observed in the ultracentrifuge at 1° is essentially unchanged, the sedimentation coefficient of the fast peak being within one Svedberg unit of that obtained with fraction S in the absence of calcium.

The electrophoretic patterns show that fraction S contains more than one type of protein. Thus the two peaks observed in the ultracentrifuge are not due to a monomer–polymer equilibrium. The electrophoretic patterns and the conditions under which fraction S was obtained suggest that β-casein is present in fraction S. Additional evidence indicating the presence of β-casein was obtained through the action of rennin on fraction S. Within one minute after rennin addition at 0°, and in the absence of calcium, colloidal aggregates will start to form. Centrifugation shows that aggregation is complete within a few minutes. The supernatant resulting from this treatment yields a sedimentation pattern having a single peak of ~1.3 *S*, the area of this peak being close to that of the slow peak observed with fraction S. This material exhibits no stabilizing action on second cycle casein. Addition of calcium to the supernatant obtained after rennin treatment produces a solution which is clear at 0° but forms a colloidal suspension at 37°. The fact that fraction S remains perfectly clear when ionic calcium is added at 37° seems to indicate that the β-casein present is being stabilized against the aggregation which calcium ions would normally produce at this temperature. Just how this stabilization is effected is obscure, for an ultracentrifuge pattern taken at 26° in the presence of calcium is close to that obtained in the absence of calcium. If the slow peak (1.3 *S*) observed at 26° is due to β-casein alone, it is β-casein present in monomeric form in the presence of calcium at 26°; at 0°, β-casein normally yields a peak of ~ 1.3 *S* even in the presence of calcium. The behavior of fraction S in the presence of calcium is receiving further attention.

Of the protein present in fraction S, 70% clearly behaves in a manner different from that of α- or β-casein. We refer to this protein as *k*-casein. It forms, at *p*H 7, a polymer whose properties are relatively insensitive either to temperature or calcium concentration. In view of the fact that *k*-casein has a mobility close to that of α-casein, its complete insensitivity to calcium is particularly interesting and significant. At more elevated *p*H values, *k*-casein dissociates into monomers similar in sedimentation coefficient to those of α- and β-casein. The S_{20} values found for *k*-casein at various levels of *p*H and temperature are summarized in Table I. The effect of rennin on *k*-casein also differentiates it from other caseins, which will be considered elsewhere,[6] as does its ability to effect the stabilization of micelles, considered below, and its phosphorus content which preliminary determinations indicate to be one-half or less that of α-casein.[11]

The Origin of *k*-Casein.—As shown above, the supernatant obtained after the first step of the second cycle (calcium addition, see Fig. 1) contains *k*-casein polymers of ~13 *S*. First cycle casein, examined at *p*H 7 and 0°, shows only two peaks, the expected β-casein monomer peak and a larger

(9) N. J. Hipp, M. S. Groves, J. H. Custer and T. L. McMeekin, THIS JOURNAL, **72**, 4928 (1950).

(10) We are indebted to Mrs. Elizabeth S. Kleiner, who has perfected this preparative procedure.

(11) Preliminary measurements of P/N ratios for current α- and β-casein fractions, fraction S and *k*-casein were very kindly carried out by Dr. Stephen M. Nagy of the Chemistry Department, Massachusetts Institute of Technology. Results on the purified fractions obtained by Mrs. Kleiner[10] will be reported subsequently.

TABLE I

SEDIMENTATION COEFFICIENTS OF k-CASEIN AS A FUNCTION OF pH AND TEMPERATURE

pH	Temp., °C.	S_{20} (Sd)
6.90[a]	1.00	13.2
6.85	21.75	13.4
6.85	31.65	14.8
11.70[b]	1.10	1.31[c]
11.70	23.30	1.27[c]

[a] K_2HPO_4–KH_2PO_4–KCl buffer, $\Gamma/2$ = 0.20, protein concn. = 0.40%. [b] K_2HPO_4–KOH buffer, $\Gamma/2$ = 0.19, protein concn. = 0.37%. [c] Mixed solution of β- and k-casein monomers.

component having a sedimentation coefficient somewhat greater than that observed for the α-casein polymers of second cycle casein ($S_{20} \sim 6.7$ *S vs.* ~ 4.5 *S*, see Table II). Apparently α-casein and k-casein exist in combination in first cycle preparations. Therefore, it seemed useful to determine more closely the point in the preparative procedure at which k-casein first appears in an independent form, our criterion being the appearance of the characteristic k-casein sedimentation peak, having an S_{20} of ~13 *S* at pH 7. Examination of a carefully prepared first cycle casein, at concentrations up to 4.5%, revealed no trace of a k-casein peak, the two peaks observed corresponded to those described above and given in Table II.

TABLE II

APPROXIMATE SEDIMENTATION COEFFICIENTS AT 1°, pH 7 AND $\Gamma/2$ = 0.15 (SVEDBERGS)

	Peak[a] I	II	III	IV	V	VI
Second cycle casein	1.3	4.5				
First cycle casein	1.3			7.5		
Fraction S (F-S)	1.3				13.5	
Second cycle casein + F-S α-/k- = 4/1	1.3			7.5		(16)
Second cycle casein + F-S α-/k- = 1/1	1.3			7.5		16
First cycle casein (treated at pH 12)	1.3		6.5			
Second cycle casein + F-S α-/k- = 4/1, (treated at pH 12)	1.3		6.5			
Second cycle casein + F-S α-/k- = 1/1, (treated at pH 12)	1.3	Broad sedimenting region				
Acid pptd. casein (treated at pH 12)	1.3		6.0			

[a] The compositions of the peaks are *largely:* I, β-casein; II, casein; III and IV, α-k-casein complex; V, k-casein; VI, k-, plus some α-casein. α-/k-ratios are by weight. The value of (16) in column VI indicates a trace. The relative area of peak I corresponded in all cases reasonably well with the relative content of β-casein in the solution, particularly if it is recognized that small amounts of β-casein interact with other caseins.

The protein solution obtained after the initial centrifugation of micelles (whey) was also examined. This solution, which contained 1.05% protein, yielded a polydisperse sedimentation pattern. The maximum ordinate of the only discrete peak visible sedimented with an S_{20} of ~3 *S* and is probably due to β-lactoglobulin. The whey protein fraction was also shown to have no stabilization action on second cycle casein, a subject which will be considered below.

It seems clear from these results and the fact that the β-casein peak is the same in first and second cycle caseins that α- and k-caseins exist as a particular α–k-complex in first cycle casein and presumably in the casein micelle. If sufficient calcium is added to first cycle casein to restore approximately the level found in skim milk, stable casein micelles are formed. However, the level of calcium used at the start of the second cycle is brought rapidly at 0° to 0.25 *M*, which is approximately eight times that found in skim milk. If first cycle casein is allowed to form stable micelles at 37° and a $CaCl_2$ concentration of 0.05 *M*, a subsequent increase to 0.25 *M* Ca^{++} results in an increase in the colloidal opacity and the formation of a small amount of precipitate. Within a few hours micelles disappear and precipitation is complete. It seems clear that, under the conditions used, the higher calcium concentration splits the α–k-complex.

First cycle casein prepared as described above was titrated at 0° with a $CaCl_2$ solution to a total Ca^{++} concentration of 0.25 *M*. The temperature was then raised to 30°, and the resulting calcium caseinate precipitate (expected to consist of both α- and β-caseins) was centrifuged out at this temperature. The supernatant, examined at 1° in the ultracentrifuge without any preliminary dialysis, yielded the typical two component fraction S pattern, a slow peak (~1.3 *S*) and the rapidly moving peak characteristic of k-casein at neutral pH.

When first cycle casein is examined electrophoretically at pH 7, two components are observed, the slower corresponding to β-casein and the more rapid being attributed to the α–k-complex. As we expect from the behavior of α-casein and k-casein alone, the mobility of this complex is close to that of α-casein. At pH 12, where the entire system dissociates into monomers, two components are observed with mobilities close to those of α- and β-caseins. These results suggest, in agreement with the behavior of fraction S, that the mobilities of α- and k-casein monomers are also similar at pH 12.

Reconstitution of the Micelle and the Interaction of k-Casein with α-Casein and β-Casein.—Purified fraction S solutions containing total protein concentrations of 0.25 to 0.40% and k-casein concentrations of 0.15 to 0.28% were mixed with second cycle caseins ranging in total protein concentration from 1.0 to 2.0% and α-casein concentrations of 0.7 to 1.4%. In one type of experiment the total protein concentration was allowed to vary and in the second it was held constant at ~1%. Aliquots of 2 ml. of stock solutions and mixtures were brought to 36.5°, and 0.1 ml. of 1 *M* $CaCl_2$ was added to each tube using the rapid mixing technique. Critical mixtures were then taken to 0° to observe the effects of a change in temperature.

As noted previously, when calcium is added to second cycle casein alone, a white flocculent precipitate forms immediately at either 0 or 37°. Addition of calcium to fraction S produces no visible change at either temperature.

The behavior of a solution containing a mixture of second cycle casein and fraction S depends prima-

rily on the relative amounts of α- and k-caseins. At 37°, as the ratio of k-casein increases, the amount of precipitate which forms on adding calcium decreases and becomes negligible when the weight ratio of α-casein to k-casein reaches four to one. The supernatants, above the precipitates which settle out in 1 or 2 hr., contain stable micelles. Greater amounts of k-casein than that required to stabilize all of the α-casein (*i.e.*, below a weight ratio of 4α- to $1k$-) lead to solutions of slightly less opacity. In going from 37 to 0°, those tubes which contain stable micelles show a reversible decrease in opacity, a decrease which is also shown by the stable micelles of first cycle casein plus calcium and by skim milk. In addition, those tubes which contain stable micelles clot on the addition of rennin in a fashion consistent with their protein concentrations and with the clotting of dilute skim milk.[6]

The weight ratio of 4 parts α- to 1 part k-casein is both just sufficient to stabilize micelles and yet is the naturally preferred ratio in the absence of calcium. At an α-/k-ratio by weight of 4/1 the ultracentrifuge pattern obtained at 0° is remarkably close to that given by first cycle casein: namely, there is the usual β-peak, a faster peak of S_{20} = 7.4 S and only a minute trace of a peak corresponding primarily to k-casein, $S_{20} \sim 16$ S (see Table II). If the α-/k-weight ratio is 1/1, three peaks are observed as indicated in Table II; the β-peak, a second peak of $S_{20} \sim 7$ S and a third peak of $S_{20} \sim 16$ S. Clearly in both cases the α-casein of second cycle casein interacts with just that amount of k-casein which will give a peak having a sedimentation coefficient of ~7.5 S. The fact that the k-casein is present in great excess in the second case apparently does not promote an α–k-interaction beyond that required to produce a peak of ~7.5 S. This in turn appears to be the minimum interaction which will form stable micelles on the addition of calcium. Since the residual k-casein peak has an S_{20} of ~16 S, we suspect that this component contains some α-casein, for the corresponding k-casein peak in the absence of α-casein is, as shown previously, relatively constant at ~13.5 S (see fraction S, Table II). In all of the interaction experiments so far described the pH was maintained between pH 6 and 7. The changes in ultracentrifuge patterns observed on mixing fraction S and second cycle casein are due primarily to the redistribution of α- and k-casein between polymer forms. That a redistribution takes place readily is not surprising, for it has been shown previously[2] that the α- and β-caseins form labile, condition-sensitive polymers.

It was felt that an examination of the interactions of the caseins proceeding from the monomer state would be important not only because the intermixing of monomers might lead to a new interaction pattern but also because monomers are observed only above pH ~ 11 and such pH values might produce changes in the proteins which would be revealed by studies of reversibility. To this end, first cycle casein and mixtures of fraction S and second cycle casein leading to α-/k-ratios of 4/1 and 1/1, all at total protein concentrations of ~1%, were titrated to pH 12 with 0.15 M KOH. After ~45 minutes, the solutions were returned to pH 7, either by titration with 0.1 M HCl or by dialysis against pH 7 phosphate buffer. In the solution having an α-/k-ratio of 1/1, the ultracentrifuge revealed a strong α–k-interaction. In the place of the discrete set of peaks previously observed, only a broad, flat sedimenting region indicating a highly polydisperse group of polymers was found. The β-peak was unchanged. In the other samples consisting of first cycle casein and a mixture whose α-/k-ratio was 4/1, the ultracentrifuge patterns after titration to pH 12 and back resembled those of first cycle casein except that the sedimentation coefficient of the α–k-peak was decreased to ~6.5 S from ~7.5 S. These data again reveal the unique characteristics of the α–/k-ratio of 4/1. These results are summarized in Table II. In all cases the addition of appropriate amounts of calcium chloride led directly to the formation of suspensions of stable micelles. On the addition of rennin the solutions clotted as expected. A sojourn at pH 12 does not therefore noticeably affect either reconstitution of micelles or clotting.

Acid Precipitated Casein.—Previously[2] we examined a commercial casein prepared by acid precipitation. This sample was of particular interest since it would not dissolve at pH 7, dissolved only with difficulty at pH 8 and then exhibited an ultracentrifuge pattern having the usual β-peak but, in place of the "α-peak," aggregates of unusual stability. After being titrated to pH 12 and returned to pH 7, "the resulting clear solution yields an ultracentrifuge pattern (1°) comparable to that obtained with soluble casein (second cycle) under the same conditions except that the α-peak sediments more rapidly ($S_{20,\alpha}$ = 6.0 S instead of 4.4 S). The S_{20} of the β-peak is 1.37."[2] In Table II these peaks are compared with others. From what has been shown above, it appears likely that the increased sedimentation coefficient of the "α-peak" is due to the fact that it actually contains both α- and k-caseins, probably in the form of an α–k-complex similar to that given by alkaline treated first cycle and reconstituted caseins. An aliquot of the same acid precipitated casein was suspended in 0.15 M KCl, titrated to pH 12 and held for 45 minutes. The clear solution was returned to pH 7.0 with 0.1 M HCl. On the addition of calcium at 37°, stable micelles formed, similar to those obtained with first cycle casein and appropriate reconstituted mixtures. These clotted, as expected, on the addition of rennin. It is clear that this acid precipitated casein differs markedly from soluble casein and that it may be converted into what appears to be first cycle casein by an appropriate alkaline treatment.

Discussion

The accumulated evidence from various sources shows that there may be obtained from skim milk a variety of phospho-proteins, namely, α-, β-, γ- and k-casein, most, if not all, of which are involved in forming casein micelles. Classically α-, β-, and γ-caseins have been prepared from acid precipitates and have been characterized not only by their phosphorus contents but by their electrophoretic

mobilities,[12] amino acid compositions[13] and recently by the manner in which phosphorus is incorporated in the protein.[14]

Evidence described here, and additional evidence regarding the action of rennin on α-, β- and *k*-caseins, clearly shows that these are separate and distinct entities. That *k*-casein also differs markedly from γ-casein, first reported by Osborne and Wakeman[15] and since isolated and characterized by Hipp, Groves, Custer and McMeekin,[9] is clear from a comparison of the abundance, electrophoretic properties and reactivity toward rennin exhibited by the two.

An examination of electrophoretic patterns allows us to estimate the relative amounts of α-, β- and *k*-caseins. Electrophoretic patterns at *p*H 12 of first cycle casein suggest that β-casein comprises ~30% of the total while α- and *k*-casein make up ~70%. Second cycle casein at *p*H 12 gives a pattern which suggests that here the α-casein is 73% and the β-casein 27%.[2] Remembering that the fraction S pattern suggests that this total protein be divided as 30% β-casein and 70% *k*-casein and that the fraction S protein is ~20% of the total, allows us to suggest that the original distribution is approximately 55% α-casein, 30% β-casein and 15% *k*-casein.

Caseins Obtained from the Acid Precipitate.—Unfractionated acid precipitated casein may contain aggregates of "α-casein" of marked stability as shown previously.[2] Since acid precipitated casein can be transformed into a system which gives essentially the first cycle casein ultracentrifuge pattern (Table II), yields stable micelles on the addition of calcium and may be clotted by rennin, it is apparent that *k*-casein has survived the step of acid precipitation. In addition, an examination of the electrophoretic pattern of acid precipitated casein which has *not* been carried to *p*H 12 (see 1) clearly suggests that *k*-casein is present in the relatively stable aggregates previously described. This is expected since α- and *k*-caseins are associated with one another in the micelle. During the process of fractionation it is apparent that *k*-casein has been carried through in the "α-casein" fraction. Thus, "α-casein" has been reported to be relatively soluble in the presence of calcium at room temperature or above and insoluble at low temperatures.[16] The solubility attributed to "α-casein"[16] at room temperature is probably due to its content of *k*-casein, thus suggesting that the "α-caseins" obtained from acid precipitates are in fact α- and *k*-caseins. The behavior of the β-casein examined here is similar to that reported by others.[9,16] We feel that many of the properties previously ascribed to "α-casein" obtained from acid precipitates, for example, in addition to solubility data such as just mentioned, the α_1–α_2-split described by Warner and others,[12,17] the distribution of phosphorus among the various bond types (Perlmann),[14] the amino acid composition[13] and the clotting behavior, (*e.g.*[18]), must be re-examined, for although α-casein and *k*-casein have similar electrophoretic mobilities, there are described here other striking differences which suggest that there must exist fundamental differences in structure and chemical composition.

The Micelle.—It is quite possible that any or all of the four proteins listed above may, in the future, be subfractionated and additional members added to the casein family. However it appears from the evidence presented, that the problem of determining the structure of the casein micelle and the physical-chemical changes involved in the process of rennin clotting require considerably more than an understanding of these as separate units. It is felt that there has been demonstrated here a clear cut interaction of α- and *k*-casein which is a prerequisite for the formation of stable micelles and, as will be detailed subsequently,[6] for the clotting of micelles with rennin.

The mechanism of the splitting action of 0.25 *M* $CaCl_2$ is under investigation. Clearly, interaction of the α–*k*-associate with Ca^{++} must lead either to an increase in inter-molecular repulsive forces (*i.e.*, through interaction with the negatively charged group of an attracting pair) or to a disturbance in surface fit which will markedly decrease short range attractive forces.[19]

Of considerable interest is the fact that the α-/*k*-weight ratio of 4/1 seems to be preferred. This ratio is selected naturally in the presence of excess *k*-casein at *p*H 7, is the minimum ratio which yields a system of stable micelles with a negligible precipitate and is the ratio which will allow a first cycle pattern to be restored after a return to *p*H 7 from *p*H 12.

If the molecular weights of α- and *k*-casein monomers are similar, the molecular ratio as well as the weight ratio of α- to *k*-casein in the peak having an $S_{20} \sim 7.5$ will be 4α- to 1*k*-. We may conclude with some assurance that essentially all of the *k*-casein monomers of first cycle casein are interacted strongly with α-casein monomers. The fact that an α-casein precipitate does not form on adding calcium to first cycle casein, while it will form when the α-/*k*- ratio is greater than 4/1 in reconstituted systems, strongly suggests that there exist few free α-casein molecules either in first cycle casein or presumably in the micelle.

Recent experiments suggest that β-casein may play a minor role in micelle stabilization. These involve mixing fraction S with purified α-casein. Stable micelles are obtained, suspensions which exhibit little change in opacity between 0 and 37° and which clot on adding rennin. These suspensions are unlike those which contain the normal amount of β-casein. Opacity changes exhibited by skim milk and by micelle suspensions obtained with first cycle casein may thus be due either to the reversible formation of the β-casein colloid previously described or to the reversible incorporation of β-casein in the micelle. Fraction S contains some β-

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(18) E. Cherbuliez and P. Baudet, *Helv. Chim. Acta*, **33**, 1673 (1950).

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casein, which makes these experiments difficult to interpret conclusively. That β-casein may play a minor role is also suggested by the fact that it is the least reactive of the group. However, β-casein does react progressively with α-casein in second cycle casein, as the temperature is increased from 4° and alone forms a colloid at higher temperatures in the presence of calcium.

That β-casein may react with the α–k-complex in the presence of calcium is indicated by the behavior of skim milk. Centrifugation of skim milk at 4° does not result in a supernatant containing all of the β-casein since in this case a large fraction of the β-casein is removed with the micelles. β-Casein could of course be partially entrapped in the micelles as a result of the fact that micelles are manufactured at a temperature near 37°. That this is not the case is suggested by the fact that the addition of calcium to skim milk at 4° shifts β-casein into a centrifugable form.

The weight of evidence so far accumulated focuses attention on the α–k-complex as being the most important single factor in micelle stabilization. Since α-casein is precipitated by calcium while k-casein is not, the formation of the α–k-complex evidently is a process which reduces the number of calcium sensitive groups so situated that calcium may act as an inter-polymer or inter-complex link. That an inter-polymer link is required is amply shown by the fact that second cycle casein may be aggregated or precipitated by very small concentrations of many di- or multi-valent cations while mono-valent cations (K^+, Na^+) do not bring about macroscopic aggregation of casein even at concentrations near saturation.[1]

The casein micelle, according to its sedimentation properties, is relatively large. Electron microscope examinations of skim milk[4,5] reveal diameters ranging from 400 to 2800 Å. The smallest micelle must contain thousands of casein monomers, yet the population of micelles is stable. The population of micelles also shows a great variation in the number of polymers per micelle. These factors indicate that the stability of the system may not be attributable to a balance of attractive and repulsive energies sensitive to micellar size, *i.e.*, critically dependent on the number of monomers per micelle. (For a discussion of micelles of this nature see Debye.[20]) We expect that, for particles as large as the casein micelles, the factors which limit size reside in the elegant series of polymerization reactions exhibited by α-, β- and k-caseins alone, in various combinations, in the presence of calcium and at different temperatures.

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