Micelle-Forming Characteristics of Monomeric and Covalent Polymeric κ-Caseins*

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ABSTRACT: Native κ-casein, whose surface activity is responsible for the formation of casein micelles, exists as SS-bonded (covalent) polymers in a wide distribution of sizes, which occasionally includes monomers. To examine structures and surface activities of monomers and polymers, and interactions with α_s- and β-caseins, the following preparations were used: standard κ-casein, prepared by a modification of the procedure of McKenzie and Wake; monomeric SH-κ-casein, *SS*-κ-casein (monomer), several covalent polymeric κ-caseins (either natural or developed by reoxidation of SH-κ-casein), carboxymethyl-κ-casein, carboxyamidomethyl-κ-casein, fractions obtained from carboxyamidomethyl-κ-casein, and standard κ-caseins with one to three ε-amino groups per monomer coupled with each of six reagents. The main results and conclusions are: (1) polymeric κ-caseins, M_n of 50×10^3 to 200×10^3, are predominantly open chains, terminated by SS-bond formation with small mercaptan molecules, (2) lateral interactions position monomer SH groups to favor κ,κ-casein SS bonding rather than chain termination, but as polymer carbohydrate content increases, the probability of chain termination increases, (3) SH-κ-casein has full surface activity; thus, SS bonds are not involved in establishing a particular monomer or polymer conformation, (4) Monomeric κ-caseins have $M_n \sim 20 \times 10^3$ and, compared to polymers, have decreased $s_{20,w}$, increased efficiency to transform large micelles into micelles of smaller size, normal, or slightly decreased α_s-casein stabilizing capacity, and decreased ability to stabilize micelles against an increase in environmental calcium. At the micelle surface, it is evidently more difficult either to insert or to remove covalent polymers compared to monomers, (5) a contribution to the association of κ-casein with α_s- or β-caseins comes from an electrostatic interaction between a positive site on κ-casein and the negatively charged acidic peptides on the other proteins. This is indicated by the effects of chemical modification of ε-amino groups of κ-casein, and effects of ions on fractionation and on κ-casein micelle surface activity, and (6) micelle formation *in vivo* may involve stabilization and size transformation of particles of α_s–β-caseinate by SH-κ-casein, followed by oxidation to covalent polymers on surfaces of micelles.

Waugh and Noble (1965) propose that in the surface of casein micelles κ-casein has a specificity such that a portion of its surface is nonreactive with respect to all protein components. Otherwise, there is a lateral specificity for other κ-casein molecules and toward the core a specificity for α_s- and β-caseins. This model has been used by others (Rose, 1965; Payens, 1966; McKenzie, 1967).

Monomer κ-caseins have molecular weights near 20,000 and contain two SH groups per molecule (Swaisgood and Brunner, 1963; Kalan and Woychik, 1965; Woychik *et al.*, 1966; Pujolle *et al.*, 1966). There are at least five species of monomer which differ in the amount of neuraminic acid and other carbohydrates attached to an SH-free macropeptide (Mackinlay and Wake, 1965; Schmidt *et al.*, 1966; Woychik *et al.*, 1966; Pujolle *et al.*, 1966; Koning *et al.*, 1966). This macropeptide is split off by action of the enzyme rennin (McKenzie, 1967). In milk, κ-casein appears to exist as disulfide-linked covalent polymers, in a distribution of sizes (Swaisgood and Brunner, 1963; Swaisgood *et al.*, 1964).

Our purpose here is to examine the mechanism by which κ-casein covalent polymer size is limited, monomer interaction and its effect on the development of covalent polymer structure, surface activities of monomers and polymers in relation to micelle distribution and stability, and electrostatic contributions to the interaction of κ-caseins with α_s- and β-caseins. An improved κ-casein preparative procedure, based on that of McKenzie and Wake (1961), is given.

Materials and Methods

Materials and Methods Previously Described. Previously described methods include laboratory distilled water, urea purification, and dialysis tubing (Waugh *et al.*, 1962).

Chemicals. Gd·HCl[1] (Eastman Organic Chemicals) was purified by the method of Greenstein and Jenrette (1942). An Ultra Pure Gd·HCl was obtained from Mann Research Laboratories. Other chemicals were analytic reagent grade.

Skim Milk and Preparation of the Caseins. Guernsey milk typed as β-casein A and α_s-casein B was used. First-cycle casein, containing essentially casein components, and fraction S, containing essentially β- and κ-caseins (Waugh *et al.*, 1962), were prepared. Preparation of α_s-casein is described by Waugh *et al.* (1970). Stock solutions containing 30 mg/ml were dialyzed against distilled water and stored at −15°.

Absorbance and Extinction Coefficients. A solvent containing 0.05 M sodium citrate at pH 7.0 was used to dilute solutions

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[1] The abbreviation used is: Gd·HCl, guanidine hydrochloride.

for absorbance determinations. Near pH 7 the $E^{1\%}_{1cm,280nm}$ has a value of 10.0 for α_s- and κ-caseins and 4.7 for β-casein (Waugh *et al.*, 1970). Corrected absorbance is determined by subtracting 1.7 times the apparent absorbance at λ 320 nm from the apparent absorbance at 280 nm. One absorbance unit, AU, is the amount of protein per milliliter required to give unit corrected absorbance at unit path.

Calcium Determination. Calcium determinations were performed by the method of Lewis and Melnick (1960).

Polyacrylamide Gel Electrophoresis. The method followed Wake and Baldwin (1961) using 6.7 M urea and 4.8% Cyanogum 41 gelling agent (Fisher Scientific Co.). κ-Casein yields a streak near the origin (Wake and Baldwin, 1961) but after reduction a series of discrete bands is obtained (Waugh, 1962; Woychik, 1964; Neelin, 1964; Schmidt, 1964). The former is a covalent polymer pattern and the latter is a monomer pattern.

Osmotic Pressure. Osmotic pressure (π) was measured at 22.5° with a Mechrolab Model 503 high-speed membrane osmometer, using Schleicher & Schuell B19 membranes and dissociating solvents: either 6 M urea–0.1 M KCl or 5 M Gd-HCl–0.1 M KCl. Measurements were made at 2, 3, 4, 5, 6, 7, and 8 mg of protein per ml. From the least-squares π/c *vs.* c relation were obtained a number-average molecular weight, M_n, and value of the first virial coefficient. Monomer samples gave zero virial coefficient and covalent polymer samples gave 4.0×10^{-7} to 9.0×10^{-7}, averaging 6.4×10^{-7}.

Sedimentation Coefficient. Studies were performed at 59,780 rpm in a Spinco Model E ultracentrifuge using schlieren optics. The results of studies in aqueous solvents will be reported as $s_{20,w}$; those in dissociating solvents (see Osmotic Pressure) as $s_{20,d}$.

Assay Centrifugation. A sample of 1.0 ml in a 15 × 75 mm tube is centrifuged at 37° for 1 min at 3800 rpm in an International Model CL clinical centrifuge. All particles having radii greater than 545 nm are removed.

Reoxidation. Certain preparations of κ-caseins have been reduced and reoxidized. In all cases complete reoxidation was indicated by a negative nitroprusside test for SH (Guzman-Barron 1951).

Stabilizing Capacity of κ-Casein. The procedure follows that of Noble and Waugh (1965). A solution containing 10 mg/ml of α_s-casein and 1 mg/ml of κ-casein is prepared at 0.03 M NaCl–0.1 M cacodylate, pH 6.6, and 37°. To 0.9 ml is added with rapid mixing 0.1 ml of 0.2 M $CaCl_2$ in a single aliquot. After 75-min incubation at 37°, the system is assay centrifuged and the fraction of the total absorbance remaining in the supernatant, U_a, is determined.

On the addition of calcium to the mixture, precipitation of Ca-α_s-caseinate tends to take place through the formation of core polymers (Waugh *et al.*, 1970). However, Ca-κ-caseinate associates with the surfaces of developing particles and, by rendering the surface nonreactive, limits the reduction of population surface area. There results a micelle size distribution which is determined mainly by the weight ratio of α_s-:κ-casein. At a weight ratio of 10, standard κ-casein produces a micelle population such that $U_a > 0.9$. As either the weight ratio is increased or the surface activity of κ-casein is decreased, average micelle size increases and the fraction of protein which appears in assay-centrifugable micelles increases, thus, U_a decreases. The stabilizing capacity of a preparation is its U_a divided by the U_a of a standard κ-casein control. We prefer this test to the widely used method of Zittle (1961) which does not specify ionic strength.

Transforming Efficiency of κ-Casein. D. F. Waugh and B. Talbot (to be published) observe that at pH 6.6, 0.016 M $CaCl_2$, and an additional ionic strength of 0.045 due to monovalent ions, micelle systems are equilibrium systems in which the size distribution is determined by the weight ratio of α_s-:κ-caseins and in which there is a low solution level of κ-casein. The addition of κ-casein to an equilibrium system decreases the weight ratio and there occurs a spontaneous decrease in average micelle size, which is termed size transformation.

To make transformation easily observed, the α_s-casein is first converted into an unstable colloid containing Ca-α_s-caseinate spheres averaging about 1 μ in diameter. A solution containing approximately 9 mg/ml of α_s-casein is dialyzed for 16 hr at 37° against 7×10^{-4} M $CaCl_2$ and 2×10^{-4} M sodium cacodylate at pH 6.6. The resulting colloid is diluted to 8 mg/ml. At 37° a final system at a weight ratio of 8 is established by mixing 4.0 ml of colloid with 1.0 ml of a solution containing 4 mg/ml of κ-casein, 0.08 M $CaCl_2$, 0.2 M NaCl, and 0.025 M sodium cacodylate at pH 6.6. If κ-casein is omitted immediate precipitation occurs. Only a small amount of added κ-casein is required to coat colloid particles and stabilize them as micelles near original size. The excess κ-casein produces size transformation which is estimated by assay centrifugation. At t = 10, 60, 150, and 300 min, aliquots are assay centrifuged and U_a determined. The U_a of the colloid alone is 0.22–0.27. For comparison the U_a for the four selected times are averaged. Standard κ-caseins gave average U_a of 0.44–0.49. The results for transforming efficiency are in the ranges 0.50–0.53, 0.44–0.49, and 0.20–0.30. The corresponding κ-caseins will be designated, respectively, as having increased, normal or decreased efficiency.

A second test for transforming efficiency employed changes in turbidity at a weight ratio of unity. Samples were examined by both tests, which were always in agreement.

Calculations. Where an average is recorded, the standard deviation of the single measurement is usually given.

Preparation of Standard κ-Casein. The most widely used method for preparing κ-casein is that of McKenzie and Wake (1961). When first used in this laboratory, final yields were 15–30% of those reported by these authors. This is probably due to the effects of temperature and calcium content. A detailed examination of experimental variables led to the following standard purification procedure.

The fraction S (~800 ml) from 1 l. of skim milk is exhaustively dialyzed at 2°, first using 0.1 M NaCl–0.1 M sodium citrate at pH 7.0, then 0.05 M NaCl, and finally 0.003 M NaCl. The calcium content is about 5.0×10^{-5} M. An equal volume of 95% ethanol is added and the temperature adjusted to 25°. This is termed FS-ethanol. A solution of 2 M ammonium acetate (NH_4OAc) in 47.5% ethanol by volume is added with stirring until precipitate formation is initiated. About 45 ml is required, and further addition has little effect on precipitate yield or contamination (fractional content of β-casein). κ-Casein precipitate is collected after 20 min, dissolved in 150 ml of 3 M urea, and exhaustively dialyzed at 2° against 0.005 M NaCl. The resulting protein solution (pH 7.1–7.4) is diluted to twice its volume with 95% ethanol, adjusted to 25°, and sufficient 2 M NH_4OAc in 47.5% ethanol added (about 2 ml) to cause precipitation. The precipitate is recovered,

dissolved in 25 ml of 3 M urea, and dialyzed exhaustively at 2° against 0.07 M KCl. Residual cream is removed by centrifugation and the preparation is stored at −15°. The *standard κ-casein* so prepared appears by gel electrophoresis to be free of impurities. Forty-nine preparations gave 1.2 ± 0.3 g of κ-casein/l. of skim milk. Approximately one-third of the protein in fraction S is recovered as κ-casein. Fifteen preparations gave an extinction coefficient, corrected for scattering, of 9.6 ± 0.4.

The conditions of the first precipitation step are 47.5% ethanol, pH 7, 25° and, approximately, 2 mg of protein per ml, 2×10^{-5} M calcium content, and 2.5×10^{-3} M sodium chloride. The effects on purity and yield of varying these conditions individually have been examined using κ-casein, β-casein, and fraction S. Of the environmental conditions, temperature and calcium content have been most important in revealing interaction characteristics of κ- and β-caseins.

In all cases, at the step where ammonium acetate is added to give precipitate, as a critical concentration is approached, turbidity slowly develops to increasing plateau levels; at the critical concentration, precipitation follows the development of turbidity; and up to ~0.03 M above the critical concentration, precipitation is more rapid, but yield and contamination remain essentially constant. A study of sodium chloride, sodium acetate, ammonium chloride, ammonium acetate, and ammonium citrate revealed that the controlling parameter is the concentration of monovalent cation, and not the particular ion or the added ionic strength.

The following refers to κ-casein and β-casein alone: (1) For standard κ-casein, between 5 and 60°, the yield is essentially constant at 70%. (2) For β-casein, yield decreases slowly with increasing temperature (from 60% at 10°) until, at a critical temperature, $T_\beta \simeq 18°$, precipitate does not form. This is in accord with other reports of temperature-dependent properties, which are generally attributed to conformational changes (Sullivan *et al.*, 1955; von Hippel and Waugh, 1955; Waugh, 1961; Payens and van Markwijk, 1963; Garnier, 1966). (3) For both κ- and β-caseins, as calcium content is increased, the critical ammonium acetate concentration progressively decreases, but to an extent 30-fold greater than that expected from ionic strength effects alone. Evidently, electrostatic repulsion is decreased by strong calcium binding. An important result is that as calcium content is increased, T_β increases.

The following refers to the first precipitation from FS-ethanol. (1) At T below T_β, the yield is about 80% and there is no significant purification. The latter has also been observed by Hill and Hansen (1963). At T just above T_β, by comparison, there is a sharp decrease in both yield (to about 65%) and β-casein contamination. As T is increased further, yield decreases (and purity increases) until at a critical temperature ($T_{\kappa\beta} \simeq 45°$) a precipitate does not form. Over the range of 5–60°, κ-casein alone precipitates. It is evident that the extent of κ- and β-casein interaction, and solubility of interaction products, increase with increasing temperature. At $T_{\kappa\beta}$, interaction products stabilize all κ-casein present.

(2) The effect of increasing calcium content is to increase both T_β and $T_{\kappa\beta}$; thus, to increase temperatures at which precipitates do not form, and to increase the temperature at which significant fractionation can be obtained. For example, under standard conditions (2×10^{-5} M calcium), $T_\beta \simeq 18°$ and $T_{\kappa\beta} \simeq 45°$, and at 10^{-3} M calcium, $T_\beta \simeq 30°$ and $T_{\kappa\beta} \simeq 58°$. Thus, at 50°, standard FS-ethanol will not yield a precipitate. However, the introduction of 10^{-3} M calcium increases $T_{\kappa\beta}$ sufficiently so that a κ-casein precipitate forms. Evidently, calcium binding must dissociate κ–β-casein interaction products.[2]

Within the range of 6.75–7.35, pH is not important in determining precipitate yield or contamination. A minimum of 43% ethanol is required for precipitate to form. As ethanol concentration is increased, there is a decrease in protein concentration which leads to a decrease in yield. Using an initial protein concentration about ten times that in FS-ethanol, Hill and Hansen (1963) find that 60% ethanol is superior to 50%, and Zittle and Custer (1963) employ 67%. One effect of ethanol is to increase ionic shielding and binding, probably since it decreases the solution dielectric constant. If methanol, ethanol, and propanol are used in otherwise comparable fractionations, the critical NH_4OAc concentrations are near 0.05, 0.02, and 0.01 M, respectively.

At precipitation steps there is a tendency preferentially to discard monomers and smaller covalent polymers. This is indicated by determination of M_n for κ-caseins before and after fractionation from admixture with β-casein, and by attempts to isolate SH-κ-casein (see below) from mixtures.

Preparation of and Results Obtained with Monomeric and Covalent Polymeric κ-Caseins. Table I summarizes properties of preparations. The first column gives the type of κ-casein and the second the type of gel electrophoresis pattern (monomer or covalent polymer). For the remaining columns, the parentheses give the number of independent preparations examined. Column 3 records average M_n and column 4 average $s_{20,w}$. Columns 5 and 6 record stabilizing capacity and size-transforming efficiency. Covalent polymeric materials have $s_{20,d}$ in the range 1.2 to 1.9 and $s_{20,d}$ increases as M_n increases. All monomeric materials have $s_{20,d}$ in the range 0.5–0.7.

Standard κ-Casein. This reference material has by definition unit stabilizing capacity and normal transforming efficiency. Its ability to stabilize ten times its weight of α_s-casein agree well with results obtained by others (Zittle, 1961; Pepper and Thompson, 1963; Mackinlay and Wake, 1965; Schmidt *et al.*, 1966; Woychik *et al.*, 1966). M_n were stable during storage at −15° for 2 months or in urea solution at room temperature during 48 hr.

κ-Casein (Sephadex Prepared). A procedure similar to that of Yaguchi and Tarrasuk (1967) and Yaguchi *et al.* (1968) was used. FCC was passed through Sephadex G-100 using a buffer containing 6 M urea and 0.005 M Tris-citrate at pH 8.6 and 23°. The first half of the broad peak which emerges near the void volume contains κ-casein alone, while the second half contains κ-casein plus increasing amounts of a protein which appears to be band 0.86 of Wake and Baldwin (1961). κ-Casein in the first half was concentrated by negative pressure dialysis against urea solution.

SH-κ-Casein. Sufficient 2-mercaptoethanol was added to standard κ-casein at pH 7.0–8.8 and 2° (at approximately I =

[2] In the preparative procedure, the important conditions of temperature and calcium content are evidently adjusted essentially to minimize κ–β-casein interactions, and to keep β-casein and association products rich in β-casein in solution. The monovalent cation concentration is then adjusted to precipitate κ-casein by reducing electrostatic repulsion between macromolecules of similar charge. Monovalent ions may also decrease κ–β-casein interactions.

TABLE I: Summary of Results for Monomeric and Covalent Polymeric κ-Caseins.[a]

Type of κ-Casein	Gel[c]	$10^{-3} \times M_n$[d]	$s_{20,w}$[e]	Stabilizing Capacity	Transforming[f]
Standard[b]	P	169 ± 34 (7)	19.6 ± 4.8 (52)	1.0	N
Sephadex prepared	P	110 ± 21 (7)			
SH-	M	19–20 (2)	11.0 ± 1.2 (12)	1.0	I (4)
CM-	M		6.0 ± 0.6 (3)	0.85	(3)
CAM-	M		12.3 ± 1.1 (3)	0.85	I (3)
SS (monomer)[b]	M	18.5–19.5 (3)	12.7 (1)	1.0	I (2)
Aqueous reoxidized[b]	P	67 ± 18 (3)	18.8 ± 4.9 (6)	1.0	N (6)
Predialyzed, aqueous reoxidized		90–100 (2)			
ε-Amino coupled	P	(Standard)	17.1 ± 2.8 (18)	0.3–0.7	D (18)
Neuraminidase treated		(Standard)	38 ± 3 (3)	0.5 ± 0.1	(3)
Fractions from CAM-κ-casein				0.6[g]	(3)
				0.9	(3)
				1.0	(3)

[a] Numbers of independent experiments are given in parentheses. [b] Two each of these preparations gave optical rotatory dispersions (Moffitt and Yang, 1956) which revealed 3% α-helix content, in agreement with Herskovits (1966). [c] Polyacrylamide gel electrophoresis pattern in 6.7 M urea: P = covalent polymer pattern, M = monomer pattern. [d] Molecular weight as determined by osmotic pressure in 6 M urea–0.1 M KCl. [e] Sedimentation coefficient in aqueous buffer, in Svedberg units. Sedimentation coefficients were insensitive to pH (6.2–7.0), ionic strength (0.01–0.06), and protein concentration (6–8 mg/ml). [f] Size transforming efficiency, N = normal, I = increased, D = decreased. [g] Stabilizations are listed in order of increasing neuraminic acid content.

0.05 and 0.04 M imidazole) to give a mercaptoethanol:SS bond ratio of 270. Reduction, complete in 5 min, was carried out for 3 hr. M_n of 19,000–20,000 are in agreement with others (Swaisgood and Brunner, 1963; Pujolle *et al.*, 1966; Woychik *et al.*, 1966). Tests for stabilizing capacity and transforming efficiency were carried out in the presence of mercaptoethanol.

CM-κ-Casein and CAM-κ-Casein. Carboxymethyl-κ-casein (CM-κ-casein) was prepared by the method of Mackinlay and Wake (1964): SH-groups are reacted with iodoacetate in the presence of 3 M Gd·HCl at pH 8.6. Carboxyamidomethyl-κ-casein (CAM-κ-casein) was prepared by the method of Woychik *et al.* (1966): groups are reacted with iodoacetamide in 4 M urea at pH 7. Materials were dialysed againt 0.05 M NaCl–0.005 M imidazole at pH 7.

SS-κ-Casein (Monomer). SH-κ-Casein at 8 mg/ml in a dissociating solvent containing 0.1 M mercaptoethanol was reoxidized during extensive dialysis at 2° against equivalent buffer free of mercaptoethanol. Samples were then dialyzed against 0.05 M NaCl–0.005 M imidazole at pH 7.

κ-Casein (Aqueous Reoxidized). SH-κ-Casein was allowed to reoxidize by extensive dialysis at 2° against 0.05 M NaCl–0.005–0.05 M imidazole at pH 7.

κ-Casein (Predialyzed, Aqueous Reoxidized). Oxygen-free distilled water was used to prepare a solution containing 0.01 M ascorbic acid, 0.07 M KCl, and 0.018 M imidazole at pH 7. Aqueous SH-κ-casein at 10 mg/ml in 0.1 M mercaptoethanol was exhaustively dialyzed against aliquots of this buffer in a closed container at 2°. Finally, the sample was dialyzed against 0.05 M NaCl–0.005 M imidazole at pH 7 in an open container, and then againt urea solution.

κ-Casein (Amino Coupled). Standard κ-casein was coupled with each of the following reagents: fluorescein isothiocyanate and 1-dimethylaminonaphthalene-5-sulfonyl chloride (Nutritional Biochemical Corp.), 1-fluoro-2,4-dinitrobenzene and succinic anhydride (Eastman Organic Chemicals), acetic anhydride (Fisher Scientific Company), and phenyl isothiocyanate (K and K Laboratories). Reagent (0.3 ml) freshly dissolved on 95% ethanol was added to 4 ml of solution containing 10 mg/ml of standard κ-casein and 0.05 M sodium phosphate at pH 8.0. The mixture was stirred at 2° for 4 hr after which it was dialyzed exhaustively at 2° against 0.05 M NaCl–0.005 M imidazole at pH 7. ε-Amino groups are the most probable site of conjugation (Fraenkel-Conrat, 1959; Fothergill, 1964; Hill and Cracker, 1968). The decrease in free amino groups per molecule, determined by ninhydrin test (Fraenkel-Conrat, 1951), was used to estimate the extent of coupling. With the colored reagents an independent estimate was made from absorbance measurements. Generally, about one-third of the added reagent molecules coupled with protein.

Each reagent was used to cover approximately 1, 2, and 3 amino groups per monomer. For all preparations, as the number of coupled groups was increased the stabilizing capacity decreased in the range from 0.7 to 0.3. A comparison of preparations in which equal numbers of groups were coupled with either phenyl isothiocyanate or fluorescein isothiocyanate revealed that the larger fluorescein led to the lower stabilizing capacity.

κ-Casein (Neuraminidase Treated). Thompson and Pepper (1962) treated κ-casein with neuraminidase and found that stabilization is still 80% of its normal value after removal of 69% of the neuraminic acid (100% release of neuraminic acid was not obtained). In experiments reported here complete release occurred. Neuraminidase, isolated from *Clostridium perfringens*, was generously supplied by Dr. S. Roseman (John Hopkins University). Its properties have been described

(Cassidy *et al.*, 1965). The enzymatic reaction and assay for neuraminic acid were kindly performed by Dr. Jacqueline Labat in the laboratory of Dr. Karl Schmid (Boston University Medical School), using the thiobarbituric acid assay of Warren (1959). The reaction was carried out for 3 hr at 37° in 0.02 M sodium cacodylate at pH 6.2. The 4-ml reaction mixture contained 10 mg/ml of κ-casein and enzyme threefold in excess of that customarily used at optimum pH. For five preparations, 2.5 ± 0.2% of dry weight was released as neuraminic acid. Others report contents of 2.4% (Alais and Jolles, 1961), 2.5% (Thompson and Pepper, 1962), 2.3% (Marier *et al.*, 1963), and 12.1–2.6% (Schmidt *et al.*, 1966).

Fractions from CAM-κ-Casein. The five prominent bands given by monomer κ-caseins on gel electrophoresis can be separated by elution from DEAE-cellulose (Mackinlay and Wake, 1965; Schmidt *et al.*, 1966; Woychik *et al.*, 1966; Pujolle *et al.*, 1966; Purkayastha *et al.*, 1967). The band of lowest mobility is free of carbohydrate; the others contain neuraminic acid and other carbohydrates in amounts which increase with electrophoretic mobility.

Three fractions were eluted using 4 M urea–0.01 M imidazole at pH 7.0 and added sodium chloride concentrations of 0.03, 0.055, and 0.09 M. Each fraction contained one or two prominent electrophoretic bands, and average mobility increased with increasing NaCl elution concentration.

Discussion

Standard κ-casein and reoxidation products of SH-κ-casein are SS-bonded covalent polymers, most of which are larger than dimers. Their M_n and $s_{20,w}$ are stable in aqueous or dissociating solvents, which suggests chain termination: either by terminal SS-bond formation with small molecules (open chains) or by grouping in SS-bonded rings. The evidence is considered to favor open chains. If ring formation were the preferred mechanism, covalent polymer size should be independent of 2-mercaptoethanol concentration. This is not the case: κ-casein (aqueous reoxidized) has a lower M_n than κ-casein (predialyzed, aqueous reoxidized). It follows also from the range of M_n that there can be no preferred ring geometry. Finally, preparations having different M_n have full stabilizing capacity: arranging open chains to give full micelle surface coverage would appear to be easier than arranging rings. In this respect it appears that SS-κ-casein (monomer) is a monomer bonded to two mercaptoethanol molecules.

The characteristics of covalent polymers depend on monomer properties. This is examined from the standpoints of reoxidation products and of the natural κ-casein in milk. In aqueous solution κ-caseins form association products whose heterogeneities are indicated by sharper trailing edges of ultracentrifuge peaks (Waugh and von Hippel, 1956; Long *et al.*, 1958; Wake, 1959; MacKenzie and Wake, 1961; Swaisgood and Brunner, 1963; Mackinlay and Wake, 1964; Garnier *et al.*, 1964; Swaisgood *et al.*, 1964; Noble and Waugh, 1965). There is agreement that the $s_{20,w}$ of each association product is relatively insensitive to pH, ionic strength, temperature, and calcium concentration, although generally (Table I), decreasing charge increases $s_{20,w}$, for both monomers and covalent polymers, and monomers have lower and more uniform $s_{20,w}$. Hill and Wake (1969) have suggested that κ-casein association products resemble soap micelles. It seems likely that association products are closed structures in the sense that product surfaces are devoid of protein–protein reactive sites. This would result if lateral κ,κ-casein specificity of interaction positions macropeptides at the surface, as they are on casein micelles. Heterogeneity would result from a combination of polymer distribution, limitations on the geometry of packing, and the requirements for lateral interaction. Lateral interactions between SH monomers appear to position SH groups so that the formation of an SS bond between κ-caseins is more probable than a bond between κ-casein and mercaptoethanol; all reoxidation products give covalent polymer gel electrophoresis patterns in which monomer bands are not evident. The same result was obtained when SH-κ-casein was reoxidized while it was on the surfaces of casein micelles.

With the exception of Beeby (1964), it is reported that κ-casein obtained from milk does not contain SH groups (Waugh *et al.*, 1960; Jollès *et al.*, 1962; Mackinlay and Wake, 1964; Swaisgood *et al.*, 1964, Nakai *et al.*, 1965; Woychik *et al.*, 1966). It is also noted that an SH-oxidizing enzyme is present in milk (Kiermeier and Petz, 1967a,b) and that normal milk serum appears to be free of small molecule SH compounds (Hutton and Patton, 1952; Zweig and Block, 1953) which might promote SS reduction or exchange. It seems likely that there is little SH-κ-casein in milk, and that the polymer distribution in normal milk is stable.

Yaguchi and Tarrasuk (1967) examine natural κ-casein covalent polymers. In their Figure 3a, a comparison of fraction A (larger κ-casein polymers) with fraction B (smaller κ-casein polymers) suggests that the larger polymers yield more κ-casein in monomer bands having lower neuraminic acid contents. This would indicate that, at the time of SS-bond formation, as the carbohydrate content of a covalent polymer increases, the probability is increased that it reacts with a chain terminator rather than with another monomer or covalent polymer. The net charge of κ-casein near neutral pH is negative (Swaisgood and Brunner, 1962; Zittle and Custer, 1963) and an increased neuraminic acid content represents an increased negative charge (Mackinlay and Wake, 1965). Electrostatic interaction might obviously affect the relative orientations of monomers, thus affect the probability of SS bonding.

Under *in vivo* conditions of covalent polymerization, lateral κ-casein interactions again appear to make κ,κ-casein SS-bond formation more probable than coupling to give *SS*-κ-casein (monomer). In the studies of whole casein by Yaguchi and Tarrasuk (1967), if such monomers were present they should be delivered from the Sephadex–urea column with monomers of α_s- and β-caseins, but monomer κ-casein bands are not discernible (fractions C and D of Figure 3a). Our electrophoresis patterns of first-cycle casein and fraction S also suggest that, generally, monomer forms are absent. However, in a few patterns, traces of κ-casein appeared in bands which indicate that *SS*-κ-casein (monomer) can form. It is possible that a non-κ-casein protein serves as a chain terminator. This appears unlikely, since all of the electrophoresis bands which stain with Amido Black have α_s-casein-stabilizing ability and are clotted by rennin (Mackinlay and Wake, 1964; Kalan and Woychik, 1965; Mackinlay and Wake, 1965; Schmidt *et al.*, 1966; Woychik *et al.*, 1966; Mackinlay *et al.*, 1966). Evidently, then, most of the κ-casein in milk is in covalent polymers, the observed range of which is from trimers (Swaisgood and Brunner, 1963; Swaisgood *et al.*, 1964) to considerably larger than decamers (to give M_n of 200,000 in Table I). The chains appear to be open and terminated by SS-

bond formation with small mercaptan molecules (possibly glutathione or cysteine).

Results using monomeric κ-caseins (Table I) show that SS bonds are not required to establish a conformation for interaction specificity. Some proteins require intramolecular SS bonds for interaction specificity, for example, ribonuclease and trypsin have no enzymatic activity after disulfide reduction (Peters and Wakelin, 1948; Sela *et al.*, 1957) or after reduction and reoxidation in the presence of dissociating agents (Haber and Anfinsen, 1962; Epstein *et al.*, 1963).

All monomer κ-caseins tested have an increased size transforming efficiency compared to polymeric materials (Table I). Rates of transformation depend on the activation energies involved in introducing κ-caseins into a micelle surface. Since the micelle surface must be essentially saturated, a κ-casein monomer should be more easily inserted than a covalent polymer. On the other hand, it should be more difficult to remove a covalent polymer, once it is present in the surface, than a monomer. That this is the case is shown by the data of Table II. As indicated, increasing the calcium concentration, after micelle formation, destabilizes micelles coated with monomer more readily than those coated with covalent polymer.

TABLE II: Stability of Micelle Systems, Using Standard κ-Casein and SH-κ-Casein, to Increased Concentrations of Calcium Chloride.[a]

	Usual Test	Increased Calcium[b]				
Total calcium molarity	0.02	0.10	0.12	0.14	0.16	0.20
Standard κ-casein	1.0	0.88	0.87	0.86	0.84	0.81
SH-κ-casein	1.0	0.79	0.74	0.71	0.68	0.60

[a] Each result is the average of four experiments, which agreed closely. [b] A micelle distribution was established as in the usual procedure. After 1 hr at 37°, additional calcium chloride was added in a 0.1-ml aliquot to give the total concentration recorded, and assay-centrifugation was carried out 1 hr later.

Data for standard κ-caseins have been examined, without success, for a correation between $s_{20,w}$ and other parameters such as M_n and, more important, variations in stabilizing capacity or transforming efficiency. This suggests that the size and/or structure of the aqueous κ-casein association product has no significant influence on κ-casein micelle forming properties. Evidently, the stability of the association product is low compared to products formed on the introduction of α_s- and/or β-caseins.

Marier *et al.* (1963) report that κ-caseins prepared by different methods vary in their content of neuraminic acid and ability to stabilize α_s-casein in micelles. A similar result has been obtained using fractions from CAM-κ-casein, and this is in agreement with the reduced stabilizing capacities of CM- and CAM-κ-caseins (Table I). Using a somewhat different test for stabilizing capacity, others (Mackinlay and Wake, 1964; Woychik *et al.*, 1966; Schmidt *et al.*, 1966) report that all κ-casein monomers have essentially the same stabilizing capacity.

The results of several studies suggest that an electrostatic interaction contributes to α_s–κ- and β–κ-casein interactions. The uniform result of chemical modification is that coupling an average of only one ε-amino group per κ-casein monomer significantly reduces stabilizing capacity and size-transforming efficiency. These are further decreased as the extent of coupling increases. Of particular interest in the series of reagents is acetic anhydride. This group should contribute least to steric effects while eliminating the charge on lysine. Woychik (1969) has shown that stabilizing capacity can be essentially eliminated by extensive trifluoroacetylation of ε-amino groups. An examination of precipitation from FS-ethanol (see above) shows that the β–κ-casein interaction product can be dissociated by the binding of calcium. There are several additional situations in which dissociation of κ-casein interaction products, either by calcium binding or by an increase in ionic strength, most readily account for experimental results: the use of high calcium chloride concentrations in the preparation of fraction S from first-cycle casein (Waugh and von Hippel, 1956), the impaired formation of micelles at high levels of sodium chloride (to be published) or calcium chloride (Waugh and Noble, 1965), and the destabilization of preformed micelles by sodium chloride (Zittle and Jasewicz, 1962) or calcium chloride (Waugh and Noble, 1965, and Table II).

It is likely that the electrostatic contribution is between amino group(s) on κ-casein and negative groups, possibly organic phosphate groups, on α_s- and β-caseins. The latter contain short phosphopeptide segments (Osterberg, 1964; Peterson *et al.*, 1958). These are placed near the surfaces of the core polymers (Waugh *et al.*, 1970) with which κ-casein interacts. Calcium is known to bind strongly to phosphate groups (Ho and Waugh, 1965) and would affect an ε-amino phosphate interaction. A contribution from hydrophobic interactions has also been suggested by Hill and Wake (1969), and hydrogen-bond formation is not excluded.

The results reported above suggest an interesting mechanism for micelle formation *in vivo*. It is that in the mammary gland, particles of α_s–β-caseinates are formed independently of SH-κ-casein. When introduced, SH-κ-casein stabilizes and transforms these particles to give the natural micelle distribution. Subsequently, oxidation on the micelle surface takes place in the presence of sufficient chain-terminating species to give the covalent polymer distribution in a particular milk. In this way superior properties would be utilized—of monomers to produce a maximum micelle population surface area and of polymers to produce population stability.

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