Core Polymers of Casein Micelles*

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ABSTRACT: The cores of casein micelles are considered to be composed of insoluble salts of α_s- and β-caseins. Core structure has been investigated by examining, for α_s-casein, β-casein and a unit weight ratio mixture of the two, at pH 6.6, the relationships between degree of monomer association and ionic strength (NaCl), divalent cation concentration (Ca(II)) or temperature. Data obtained were sedimentation coefficient, $s_{20,w}$, and reduced viscosity, $[\eta]$. For β-casein and the mixture at 37°, with calcium absent, as NaCl increases, $s_{20,w}$ increases and $[\eta]$ decreases. Both plateau at ionic strengths above 1.5 until precipitation occurs; near saturation for β-casein and at about 3.5 M NaCl for the mixture. The degree of association also increases as the temperature is increased. Plateau values for $s_{20,w}$ and $[\eta]$ have been observed just prior to precipitation over a temperature range near 23° for β-casein at 0.048 M NaCl and 0.015 M $CaCl_2$ and for the mixture at 0.048 M NaCl and 0.009 M $CaCl_2$. α_s-Casein behaves differently. For example, it precipitates near ionic strength 0.6 (NaCl) in the absence of calcium and plateaus in $s_{20,w}$ and $[\eta]$ are not observed. Plateaus in both $s_{20,w}$ and $[\eta]$ just before precipitation suggest that polymers have arrived at limiting average values of size, shape, and solvation. Application of the equations of Simha and Perrin indicates that limiting polymers are spherical, have a maximum degree of association near 30, and have solvations ranging from 2.2 to 4.4 g of water per g of protein. The degrees of association calculated using $s_{20,w}$ and $[\eta]$ are in agreement with those obtained independently by Archibald analysis of polymer weights.

The limiting (core) polymer is considered to contain radially arranged anisometric monomers. It is proposed that monomers consist of elongated bodies of low net charge having characteristic solvent accessible acidic peptides attached near their ends. Electrostatic free energy is minimized by placing the acidic peptides near the polymer surface. When the acidic peptides are sufficiently discharged by ion shielding or binding, interpolymer interactions allow precipitation (core formation) to occur. A comparison of the behavior of individual components and the mixture shows that α_s- and β-caseins intermingle at the molecular level in the mixture and that polymers of α_s-casein have a larger interpolymer attractive energy than those of β-casein.

It has been proposed (Noble and Waugh, 1965; Waugh and Noble, 1965) that casein micelles of bovine milk are composed of variable size cores of insoluble salts of α_s- or β-caseinates covered by a coat of uniform thickness containing κ-casein. This structure has been accepted by McKenzie (1967), Payens (1966), and Rose (1965). The κ-casein in the coat has been found to be a single molecular layer in thickness (D. F. Waugh and B. Talbot, in preparation).

We would expect, then, that the properties of the micelle core would be determined largely by the properties of α_s- and β-caseins and their interactions, among themselves and with their environments, in the development of precipitates. In milk, the development of core structure evidently involves a complex set of interactions between α_s- and β-caseins and at least calcium, magnesium, phosphate, and citrate (Davies and White, 1960). The complexity can be greatly reduced: for some time, most studies of micelle formation have been carried out using calcium as the driving ion. In fact, micelles can be made with most divalent cations (Waugh, 1961) and the size distributions obtained with some, including calcium, have been shown to resemble closely size distributions found in milk (Adachi, 1963).

We report here studies of the course of the development of polymers up to the point of precipitation in systems containing α_s- or β-caseins, or mixtures of the two. At this time, studies of sedimentation and viscosity characteristics of polymers have been carried out using temperature, calcium concentration, and ionic strength as variables. The results suggest that a polymer of limited size forms prior to precipitation. Models for monomer structure, polymer structure, and the interaction of polymers in the formation of precipitates are derived.

Numerous studies by others, carried out in the absence of calcium, have shown that α_s-casein (Ashworth, 1964; Driezen *et al.*, 1962; Ho and Chen, 1967; Krescheck *et al.*, 1964; McKenzie and Wake, 1959; Payens, 1968; Payens and Schmidt, 1965, 1966; Schmidt and Payens, 1963, 1964; Schmidt and van Markwijk, 1968; Swaisgood and Timasheff, 1966; Thompson and Pepper, 1964a), β-casein (Ashworth, 1964; Halwer, 1954; Krescheck *et al.*, 1964; McKenzie and Wake, 1959; Payens, 1968; Payens and van Markwijk, 1963; Sullivan *et al.*, 1955; von Hippel and Waugh, 1955), and mixtures of α_s- and β-caseins (Ashworth, 1964; Payens, 1968)

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interact and polymerize (associate) to different degrees under various conditions of pH, ionic strength, and temperature. These studies were carried out at relatively low ionic strength (up to $I = 0.25$). Under these conditions, casein polymers differ strikingly in sedimentation and viscosity properties from polymers which are present just before precipitation. For this reason, the relationships between core formation and polymers formed at $I \leqslant 0.25$ in the absence of calcium are obscure.

Materials and Methods

Materials and methods previously described are laboratory-distilled water, dialysis tubing, chemicals, and the preparation of first cycle casein which contains essentially all of the casein of milk, free of whey protein (Waugh *et al.*, 1962). Solvations of precipitates were determined by the procedure of Waugh and Noble (1965).

Skim Milk. Milk obtained from individual Guernsey cows known to produce only the B variant of α_s-casein and A variant of β-casein was cooled at once to 4°, skimmed by bucket centrifugation at 800*g* for 45 min, frozen, and stored at −15°.

Urea and Urea Buffers. Analytical reagent grade urea was dissolved to give a 4.5 M solution, filtered to remove colloidal particles, passed through ion exchanger to replace divalent cations with sodium ions, and acidified to destroy cyanate (Waugh *et al.*, 1962). Buffer I (B-I) was obtained from this by adding imidazole to give 0.01 M and titrating to pH 7. B-I has an ionic strength determined by additions of HCl and imidazole and the salt content of the solid urea. Urea from J. T. Baker Co. produced B-I having an ionic strength near 0.04 while urea from Mallinckrodt Co. gave a B-I ionic strength near 0.02. The amount of salt present in each B-I was determined by conductivity measurements made in an ice bath and at 25.0 ± 0.1°. Sodium chloride was then dissolved to give a desired total ionic strength, which will be designated (*e.g.*, B-I 0.12). Urea (9 M) was processed a similar way.

Polyacrylamide Gel Electrophoresis. Two systems were used. The first contained 310 ml of 9 M urea, 40 ml of buffer consisting of 0.76 M Tris adjusted to pH 8.6 with solid citric acid, 20 ml of water, 20 g of Cyanogum 41 gelling agent, 0.4 ml of Cyanogum 41 catalyst (American Cyanamide Co.) and 0.3 g of ammonium persulfate. In the second, the 40 ml of buffer contained 0.05 M magnesium acetate, 0.25 M boric acid, and 0.25 M Tris. Proteins were electrophoresed for 16 hr at 2° at approximately 10 V/cm. Bands were stained by immersion for 4 hr in 0.1% Amido Black 10B (Hartman-Leddon Co.) in 5% acetic acid solution. Gels were exhaustively washed in 1% acetic acid, then with water for 48 hr, after which bands were cut out and adsorbed dye was taken into solution with 3% aqueous ammonia. The amount of released dye was determined by absorption at 613 nm using a Beckman DU spectrophotometer.

In the second procedure, employing magnesium acetate and boric acid, proteins other than α_s- and β-caseins (impurities) appear between the slot and β-casein, a circumstance which facilitates detection and determination. In the first system, one impurity appears between the positions of β-caseins B and C.

Protein Concentration. This is most conveniently determined from absorbance measurements. A correction is made for scattering by subtracting 1.7 times the apparent absorbance at λ 320 nm from the absorbance at λ 280 nm. Waugh (1969) has summarized the values determined from the absorptivity of the caseins. We will use values of 10.0 for α_s-casein and 4.7 for β-casein. The final concentrations will generally be reported in units of mg per ml. In crude samples, for which an average absorptivity is not known, the amount of protein will be specified by total absorbance units (AU).

Preparation of α_s- and β-Caseins. The starting material for fractionation was first cycle casein dialyzed against 0.2 M KCl (instead of 0.08 M sodium acetate as used by Waugh *et al.* (1962)).

The procedure, developed by Dresdner (1965), was designed to accomplish fractionation of individual β-caseins from animals having the most frequent genetic typings AA, AB, and AC, and the less frequent BB and CC combinations. This permitted a comparison of the variants obtained from heterozygous animals. The main problems encountered when attempts are made to obtain pure β-caseins from heterozygotes are that the critical elution ionic strengths lie in a narrow range, as found for α_s-casein, and impurities may be present which elute at ionic strengths close to those of the β-caseins: impurity I-1 just before β-caseins A and B, I-2 and I-3 between β-caseins B and C, and I-4 just after β-casein C. It is difficult to fractionate when these impurities are present. For animals giving AA, AB, and BB combinations, I-2 and I-3 are absent; apparently their synthesis is linked to the synthesis of the C component and purification is simplified. I-1 is found always to be present in crude β-casein if the initial calcium treatment of first cycle casein (splitting) is carried out according to the procedure of Waugh *et al.* (1962). Conditions were not found for separating I-1 from A or B components (or A and B components from each other) since all three elute at nearly the same ionic strength. It was found that I-1 has the solubility characteristics of the high phosphorous "α_s-casein fractions" (Waugh *et al.*, 1962) and that it can be eliminated by discarding a small amount of precipitate which forms when first cycle casein is adjusted to 0.25 M $CaCl_2$ (split) and allowed to stand at 2°. This precipitate also contains an impurity which is difficult to separate from α_s-casein if it is present in crude α_s-casein. The procedures for obtaining α_s-casein B (or AB) and β-casein A (or AB or B) are as follows.

First cycle casein from 1 l. of skim milk was thawed, brought to 900 ml with water, and adjusted to pH 7.4 using 2 M Tris at pH 9. Sufficient 5 M calcium chloride was added at 0° to give 0.25 M and the system was kept near pH 7 by simultaneously adding 2 M Tris at pH 10. A precipitate formed at once. The system was placed at 2° overnight, after which the supernatant was recovered and the 2° precipitate was discarded. The supernatant was now raised to 37° and centrifuged at this temperature for 30 min at 17,000 rpm using the no. 20 head of the Model L Spinco ultracentrifuge. The supernatant may be used to prepare κ-casein.

The 37° precipitate was separated into crude α_s-casein and crude β-casein using a modification of the procedure of Hipp *et al.* (1952a). The precipitate was dissolved in 350 ml of 9 M urea at 23° and adjusted to pH 4.8 with 2 M HCl. The addition of 600 ml of water and 10-min stirring produced a precipitate of crude α_s-casein and a supernatant containing

TABLE I: Compositions of Materials Which Appear During Fractionation.[a]

	α_s-Casein (%)	β-Casein (%)	Impurities (%)
2° precipitate	50	14	36
37° precipitate	37	45	18
Crude α_s-casein	64	10	26
Crude β-casein	13	71	16

[a] κ-Casein essentially absent in all materials.

crude β-casein. The precipitate was compacted by centrifugation. The precipitate of crude α_s-casein was dissolved in 350 ml of 9 M urea, adjusted to pH 7.0 with 2 M Tris, and stored at −15°

Table I gives the relative amounts of α_s-casein, β-casein, and impurities found in the materials referred to above using the second polyacrylamide gel electrophoresis procedure and dye content determination.

Column fractionation of both proteins employed DEAE-cellulose having a capacity of 0.83 mequiv/g (lot no. 1646, Schleicher & Schuell). α_s-Casein was obtained from crude α_s-casein according to the three-column procedure of Waugh *et al.* (1962). After applying 5000 AU of crude α_s-casein in 2 l. of B-I 0.145, the first column was washed with 6 l. of the same buffer and the wash was discarded. After this, 18 l. of B-I 0.1775 was passed through the first column and a second retarding column previously equilibrated with the same buffer. The output of the retarding column was diluted with distilled water and its α_s-casein content adsorbed to a collecting column, from which it was eluted using B-I 0.5. Approximately 2 g of α_s-casein was recovered from the collecting column.

The supernatant containing crude β-casein was adjusted to pH 7 with 2 M Tris and stored at −15°. For fractionation, a single column of DEAE-cellulose 18.5 cm in diameter and 5 cm long was washed (see Waugh *et al.*, 1962) and equilibrated with B-I at 12°. Crude β-casein was diluted with B-I to give 3000 AU in 2 l. and this was brought to 12° and applied to the column at a flow rate of ~50 ml/min. Columns were monitored with a Cary Model 11 spectrophotometer at 280 nm. After applying the protein, 17 l. of B-I 0.045 was passed at 25 ml/min and the effluent, which contains a variety of impurities, was discarded. β-Casein A was then removed using 10 l. of B-I 0.095. The critical elution ionic strength of β-caseins A or B and those of α_s-caseins and other impurities are sufficiently close so that accurate control of ionic strength must be achieved and large volumes used to elute at a low (maximum 1.30 AU/ml) level of solution protein. When β-casein was eluted, the effluent was diluted with an equal volume of cold distilled water and β-casein was adsorbed to a collecting column of DEAE-cellulose of the same size as the fractionating column. Elution in a small volume from the collecting column was accomplished using B-I 0.50. β-Caseins were then dialyzed at 4° using four changes of 7 l. each of neutral distilled water over a period of 2 days. The dialyzed protein was freeze dried and stored in polyethylene bottles at 20°. The collecting column gave a yield near 2 g of β-casein.

Details of more complex fractionation procedures used when C component is present are given by Dresdner (1965). In this case the selection of an elution ionic strength for fractionation is strongly dependent on the capacity of the DEAE-cellulose. Pion *et al.* (1965) and Thompson and Pepper (1964b) have also published procedures to obtain β-caseins from homozygous animals.

The results obtained by these sources and by us are in general agreement, where the same properties were examined, and may be summarized as follows. The β-caseins are free of carbohydrate, sulfhydryl, and disulfide. The measured absorptivities range between 4.5 and 4.7, a range which is probably produced by experimental error. The absorptivities suggest four tyrosine and one tryptophan residue per molecule of mol wt 24,100. The C-terminal residue is isoleucine. β-Caseins A and B contain five phosphorous atoms per molecule and β-casein C, four atoms.

pH. A pH of 6.6 has been used throughout. This is close to the natural pH of milk and is, further, a pH at which reconstituted systems have equilibrium characteristics (D. F. Waugh and B. Talbot, in preparation).

α_s- and β-Casein Mixture. In all mixed systems, a 1:1 weight ratio of α_s- to β-caseins has been used. This ratio is close to the natural α_s-:β-casein ratio in milk.

Ionic Strength. Protein solutions are used which contain varying amounts of calcium chloride and sodium chloride. The reported ionic strength, I, of each solution, will refer to the amount of sodium chloride present and the amount of calcium chloride used in each case will be specified separately.

High Salt Concentrations. Solutions which contain sodium chloride in excess of 0.25 M will be considered as having a high salt concentration. No $CaCl_2$ is added and the NaCl is added by mixing the proper volumes of 5.0 M NaCl, an aqueous solution of protein and 0.10 M NaOH to give the final specified concentrations of NaCl and protein at pH 6.6. The contribution of sodium hydroxide to adjust pH is negligible.

Low Salt Concentrations. A slightly different procedure was followed when the sodium chloride was less than 0.25 M. For these cases, a concentrated protein solution (25 mg/ml) was dialyzed against a solution of the proper ionic strength and sufficient 0.10 M cacodylate was added to an aliquot to give a final concentration, after dilution, of 0.002 M. When $CaCl_2$ is added to these solutions, there is hydrogen ion release through calcium binding and 0.10 M NaOH was added to adjust the pH to 6.6. This addition increases with increasing $CaCl_2$ concentration. Finally, an appropriate sodium chloride solution was added to bring the sample to its final specified protein and salt concentrations.

Ultracentrifugation. A Spinco Model E analytical ultracentrifuge equipped with schlieren optics was employed in ultracentrifugal analysis. The temperature was controlled by the RTIC unit to within ±0.2°. Sedimentation coefficients were determined from the best straight line, obtained by least-squares analysis, on a plot of log r_{max} *vs.* time. The distance from the center of rotation to the maximum ordinate, r_{max}, was used at all times. The values obtained will be reported in Svedberg units (S). All sedimentation coefficients have been corrected to water at 20°.

The molecular weights of certain polymers were determined by the Archibald (1947) technique. In this case, $(1/cr)(\partial c/\partial r)$

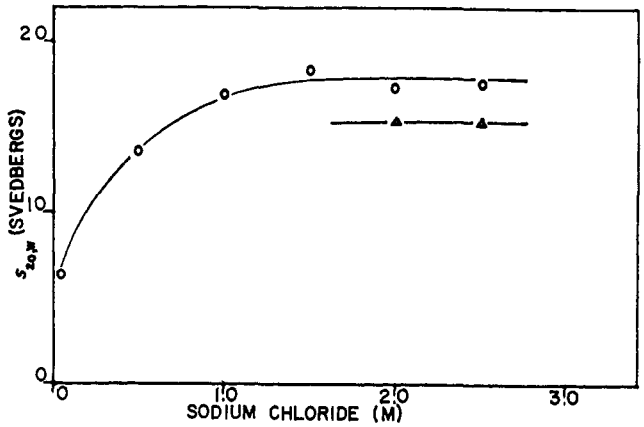

FIGURE 1: Sedimentation coefficient *vs.* sodium chloride concentration for β-casein (○) and unit weight ratio mixtures of α_s- and β-caseins (△). Concentration in each case was 10 mg/ml, temperature was 37°, and pH 6.6.

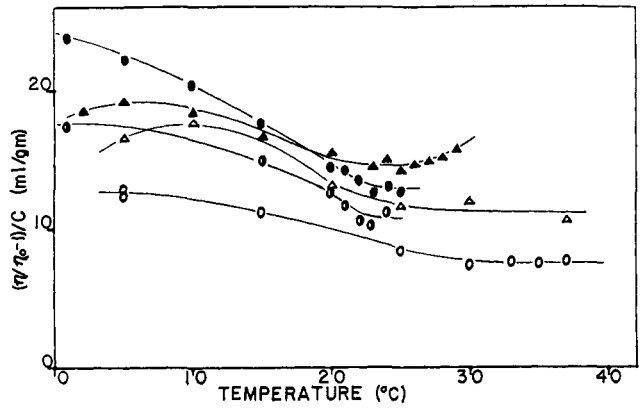

FIGURE 2: Reduced viscosity *vs.* temperature. Circles refer to β-casein as follows: (○) 10 mg/ml, I = 2.0; (◑) 5.8 mg/ml, I = 0.048 and 0.015 M calcium; (●) 11 mg/ml, I = 0.048 and 0.015 M calcium; triangles refer to unit weight ratio mixtures of α_s- and β-caseins as follows: (△) 10 mg/ml, I = 2.0; (▲) 9.8 mg/ml, I = 0.048, 0.009 M calcium. All were at pH 6.6.

was plotted *vs.* r and extrapolated to the meniscus. The concentration, c, was found by numerical integration of the gradient curves. A double-sector cell was used so that the base line was visible and the gradient height could be measured easily. More consistent results were obtained by employing a modification proposed by Ehrenberg (1957) in which a high centrifugal field is used until the top of the peak is visible at the meniscus. This gives an almost horizontal extrapolation to the meniscus and reduces the error due to inaccuracy in establishing the meniscus position.

Viscosity. Ostwald, Cannon, and Fenske viscometers were used, immersed in a water bath held at constant temperature by means of a Thermistemp Model 71 temperature controller manufactured by Yellow Springs Instrument Co., Inc. The time required for a buffer (220–460 sec) or a particular sample to flow through the viscometer could be duplicated to within a few tenths of 1% in all cases. Generally, duplication was of an order of magnitude better than this. Although all measurements pertaining to a particular sample were usually made on the same day, less than a 1% difference was found for any given sample at a given temperature on different days.

In the following, the symbol $[\eta]$ will refer to $((\eta/\eta_0) - 1)/c$, where η and η_0 are the solution and solvent viscosities, respectively, and c is concentration in grams per milliliter. Since interest centers around association products, extrapolation to infinite dilution to obtain intrinsic viscosity has not been carried out. In applying eq 1, $[\eta]$ is used in place of intrinsic viscosity.

Results

β-Casein. As the concentration of sodium chloride is increased in a protein solution containing 10 mg/ml of β-casein, s_{37} corrected to $s_{20,w}$ (Figure 1), increases until the sodium chloride concentration is near 1.5 M. Above this level, $s_{20,w}$ plateaus until the protein salts out above 4.5 M, which is near the saturation level of sodium chloride. An ionic strength of 2.0 was chosen for an examination of the temperature dependency of viscosity (Figure 2). The reduced viscosity decreases with increasing temperature and plateaus at temperatures above approximately 20°.

Some important information can be gained from a consideration of Figure 3 which shows the concentration dependence of sedimentation for β-casein at I = 2.0. If a linear relationship such as $s = s^0(1 - kc)$ is assumed, k is calculated to be 0.20 dl/g. This is close to the value found for compact, globular proteins (Harrington *et al.*, 1956; Kegeles and Gutter, 1951; Miller and Golder, 1952; Slattery, 1965). Sedimentation patterns from the analytical ultracentrifugation at $I < 1.5$ show, in each case, a sharp boundary and high concentration dependence, such as one would obtain with a highly asymmetric molecule. This self-sharpening of the boundary disappears when the region of constant $s_{20,w}$ is reached. Only the single peak is observed in these patterns. An Archibald analysis at 37°, I = 2.0 gives a molecular weight for the polymer of about 820,000. Using a value of

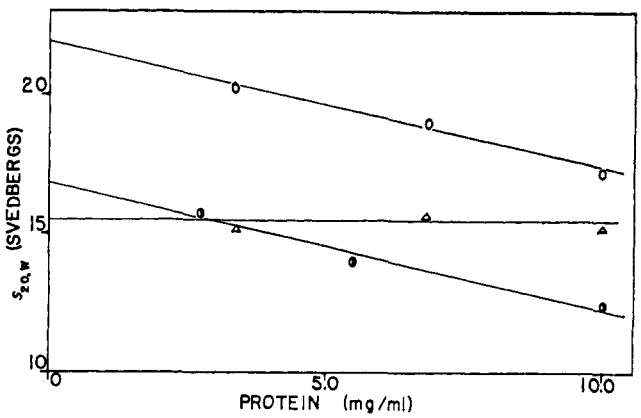

FIGURE 3: Sedimentation coefficient *vs.* protein concentration. Circles refer to β-caseins as follows: (○) I = 2.0, 37°; (◑) I = 0.048, 0.015 M calcium, 23°. The triangle refers to a unit weight ratio mixture of α_s- and β-caseins at I = 2.0, 37°. All were at pH 6.6.

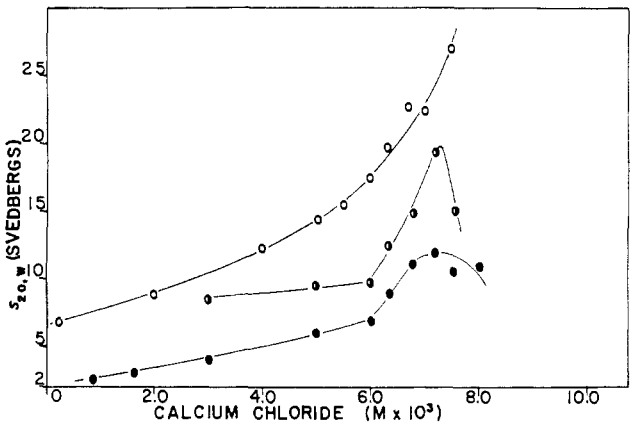

FIGURE 4: Sedimentation coefficient *vs.* calcium chloride concentration. (○) β-Casein at 10 mg/ml, I = 0.048, 37°. The symbols ● and ◑ refer to the slow- and fast-moving peaks in solutions of α_s-casein at 15 mg/ml, I = 0.028, 37°. All were at pH 6.6.

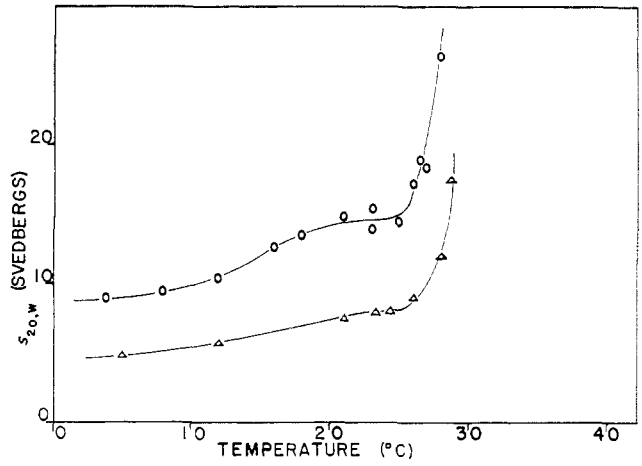

FIGURE 5: Sedimentation coefficient *vs.* temperature. (○) β-Casein at 11 mg/ml, I = 0.048, 0.015 M calcium. (△) Unit weight ratio mixture of α_s- and β-caseins at 9.8 mg/ml, I = 0.048 and 0.009 M calcium. All were at pH 6.6.

24,100 for the monomer molecular weight (Sullivan *et al.*, 1955) gives an approximate degree of association of 34.

Similar effects to those already described can be obtained at a low ionic strength by the addition of calcium (II) ions to bind to the protein and reduce the effective charge on the molecule. At 37° and I = 0.048, the $s_{20,w}$ increases as the calcium ion concentration increases, as shown in Figure 4. Precipitation occurs between 0.007 and 0.0075 M calcium. In all systems where calcium was used as the driving ion, a small fraction (<5%) of the protein was present in a peak with $s_{20,w}$ near 1.5 S.

We chose to study the effects of temperature at I = 0.048 using 0.015 M calcium ion concentration. This concentration was found by Noble and Waugh (1965) to be satisfactory for micelle formation in situations where κ-casein is present. Figure 5 shows the manner in which the sedimentation coefficient increases as the temperature is increased. Here again, the polymer increases in $s_{20,w}$ until a constant value is reached between 21 and 25°. It then increases again until precipitation occurs between 26 and 27°. The reduced viscosity also reaches a limiting value between 23 and 25° (Figure 2). For this polymer at 23°, according to Figure 3, the concentration dependence of sedimentation is slightly larger than for the polymer which occurs at 37° and I = 2.0 (Figure 3). Archibald analysis gives a molecular weight of about 650,000 or an approximate degree of association of 27.

1:1 α_s- and β-Casein. Results of studies comparable with those with β-casein using a 1:1 weight ratio of α_s- and β-caseins are shown in Figure 1 ($s_{20,w}$ *vs.* I), Figure 2 ([η] *vs.* T), Figure 3 ($s_{20,w}$ *vs.* c), and Figure 5 ($s_{20,w}$ *vs.* T). Evidently, just before precipitation $s_{20,w}$ and [η] arrive at plateau values. By Archibald analysis the polymer at I = 2.0 and 37° has a molecular weight of about 640,000 or 25 monomer units/polymer using an average monomer weight of 25,600 daltons. At I = 0.048, 0.009 M Ca and 23°, the polymer has a molecular weight of about 480,000 or a degree of association of 19. The α_s–β-casein system precipitates near 3.5 M sodium chloride.

α_s-Casein. A much more complex situation arises when an attempt is made to conduct the same types of experiments on α_s-casein alone. The protein precipitates, with no calcium ion present, at between I = 0.5 and I = 0.6. At I = 0.5 and 37°, the sedimentation pattern shows a large degree of heterogeneity which forestalls any attempt at analysis.

Figure 4 shows the effect of calcium addition on the sedimentation coefficient for α_s-casein at I = 0.028. At first $s_{20,w}$ increases slowly with a single peak present. When the calcium ion concentration is near 0.003 M, a second, faster peak appears. Both peaks increase in $s_{20,w}$ but the area of the fast peak increases while that of the slow peak decreases. Before precipitation, at just above 0.007 M Ca, there is a rapid increase in $s_{20,w}$. After precipitation, the large polymers have entered the precipitate and those which remain have a lower sedimentation coefficient.

For systems in which plateaus are observed, limiting values of $s_{20,w}$ and [η] and degrees of association calculated from measured polymer weights are given in Table II.

Discussion

The assumption is made that important micelle core properties can be determined from the above characterization of the interactions of α_s- and β-caseins, individually and with each other. Certain relevant monomer characteristics are summarized as follows.

α_s-Casein has a molecular weight near 27,000 (Driezen *et al.*, 1962; McKenzie and Wake, 1959), contains nine phosphorous atoms per molecule and has a high fractional content (0.34) of the large nonpolar side chains (Val, Leu, Ile, Phe, Trp, Pro) (Gordon *et al.*, 1965; Hipp *et al.*, 1961). One of the important features of this molecule is the presence of a short nonterminal peptide chain of 35 amino acid residues which contains 7 of the 9 organic phosphate groups, 11 carboxyl groups, and 2 amino groups (Osterberg, 1964). It is most likely that the phosphate groups are serine monoesters (Anderson and Kelley, 1959; Hofman, 1958; Kalan and Telka, 1959a,b; Osterberg, 1961). At pH 6.6, I = 0.05 and 20°, the α_s-casein monomer carries a net charge of about −22 (Ho and Waugh, 1965). From its structure the acidic peptide is expected to account for essentially all of the molecular net charge.

TABLE II

Casein	I	Temp (°C)	Ca	$s_{20,w}$ (S)	$[\eta]$	G	$n_{s,\eta}$	$r_{s,\eta}$	n_{arch}	r_{arch}
β	2	37	0	18.0	7.3	2.2	31	95	34	104
$\alpha + \beta$	2	37	0	15.2	11.5	3.9	28	108	25	96
β	0.048	23	0.015	14.6	12.8	4.4	30	114	27	103
$\alpha + \beta$	0.048	23	0.009	8.0	14.6	5.1	12	88	19[a]	139
β	0.048	37	0.007	27.5						
α_s	0.048	37	0.0072	19.5						

[a] Carried out at 26°. The degree of association is expected to be higher than that calculated for 23°.

β-Casein has a molecular weight near 24,000 (Payens and van Markwijk, 1963; Sullivan *et al.*, 1955), contains four to five phosphorous atoms per molecule and the calculated nonpolar side-chain frequency is 0.45 (Peterson *et al.*, 1966; Pion *et al.*, 1965). All of the phosphate groups are present in a terminal peptide chain of 24 amino acid residues which contains in addition 5 carboxyl groups and 2 arginines both of which are terminal in the peptide (R. F. Peterson, unpublished data; Peterson *et al.*, 1958). Our titration data show that at pH 6.6, $I = 0.048$ and 25°, the monomer carries a net negative charge of -13 ± 1. This is in close agreement with the results of Hipp *et al.* (1952b). From its structure the acidic peptide is again expected to account essentially for all molecular net charge. Both α_s- and β-caseins are free of carbohydrate, sulfhydryl, or disulfide groups.

The data to be correlated by appropriate monomer structures and association characteristics are ion binding and solvation and, in addition, for proteins in solution, viscosity, and sedimentation behavior. Electrostatic free energy, ion binding, and solvation have been studied using pure components and mixtures and will be examined elsewhere. An important conclusion arising from these studies is that the acidic peptides of α_s- and β-caseins are freely available to the solvent. Similar models are proposed for these proteins: compact bodies, stabilized by hydrophobic interactions, to which are attached solvent accessible acidic peptides. For convenience bodies will be considered to be ellipsoids of revolution. For β-casein the acidic peptide, since it is attached at only one of its ends, could have considerable conformational freedom in the solvent; for α_s-casein (see Figure 6) the peptide, attached at both ends, must form a part of a loop and thus have restricted conformations. It is likely that the body would have the usual mode of distribution of side chains: ionic and polar on the surface while nonpolar side chains are internal (Perutz *et al.*, 1965). The high nonpolar side-chain frequencies (0.4 and 0.5 for α_s- and β-casein bodies) suggest, however, that whatever the asymmetry of the body, it is not possible to pack all of the nonpolar side chains internally. Some, at the surface and available for intermolecular interaction, are considered to be the main source of attractive interaction energy for the development of polymers. The acidic peptides would constitute, on the other hand, the main source of repulsive energy to limit the degree of association. In this model the charged ends are arranged radially (Figure 6b) to place them as far apart as possible and thus minimize electrostatic free energy. The degree of association should be increased by any factor which tends to increase hydrophobic bonding, such as increased temperature, or which reduces the effective negative charge on the molecule. Effective charge reduction and concomitant collapse of acidic peptides can be accomplished by binding of positive ions or by a simple increase in ionic strength which produces an increase in shielding. The degree of association increases until a complete spherical rosette is formed and additional monomers cannot be introduced without increasing the free energy of formation of the polymer. The polymer has a structure like that of a spherical soap micelle (Shinoda *et al.*, 1963) and essentially as a result of the same types of interaction characteristics. It is noted that the radial arrangement of monomers in the complete polymer places most of the occluded solvent near the polymer surface where it can accomodate acidic peptides and solvent volumes they might dominate. A sufficient reduction in repulsion could allow polymers to precipitate. The introduction of calcium to increase the degree of association introduces, as will be evident, complications which may be due to calcium cross-linking. For this reason, effects of ionic strength are examined first.

A number of studies of polymerization have been made by others, using ionic strength (sodium chloride) up to 0.25. In all of these (references are given earlier) it was found that the degree of association increases with ionic strength and is generally low (up to ~10). It should be noted that the various reports on β-casein are not as consistent as those on α_s-casein. For instance, Payens and van Markwijk (1963) suggest that β-casein is a threadlike polymer of ~100 mono-

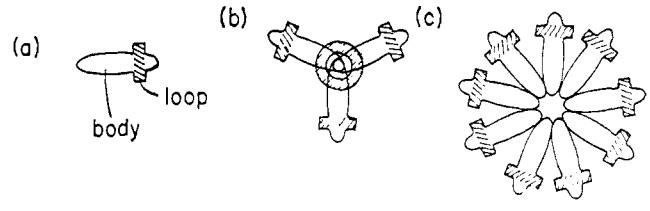

FIGURE 6: Monomer and polymer models for α_s-casein. (a) Monomer, (b) tetramer, and (c) planar representation of complete core polymer. The acidic peptide dominates the region indicated as a loop.

mer units in barbituate buffer, pH 7.5, 13.5°, and $I = 0.2$. Our observations are not in accord with this and we can only conclude that there must be considerable path dependency involved in these association reactions. Possibly this path dependency is similar to that suggested by Noble and Waugh (1965) for interactions of κ-casein with α_s- and β-caseins in the absence of calcium. Payens and van Markwijk (1963) do observe, as we have confirmed, that sedimentation boundaries are hypersharp and show a high concentration dependence. Although sedimentation and viscosity data are difficult to interpret quantitatively under these conditions, the combined data are in agreement that, with increasing salt, polymer weight increases and frictional resistance decreases due to axial ratio and/or solvation decrease.

Where both $s_{20,w}$ and $[\eta]$ plateau, the situation may be more easily examined since this behavior suggests that polymers have arrived at limiting average values of size, shape, and solvation. For generality, assume that the polymers themselves have an axial ratio. Intrinsic viscosity, solvation, and viscosity increment are related by eq 1, where 0.74 is

$$\nu^*(0.74 + G) = [\eta] \tag{1}$$

approximately the reciprocal of the protein density and G is the amount of solvent, in grams of water per gram of protein, associated with the polymer hydrodynamic unit. ν^*, the viscosity increment, is related to the axial ratio of the hydrodynamic unit, as an ellipsoid, by Simha's equation (Simha, 1940; Mehl *et al.*, 1940).

The sedimentation coefficient is related to the frictional coefficient of the polymer hydrodynamic unit by

$$s = \frac{nM(1 - \bar{v}\rho)}{Nf} \tag{2}$$

where n is the degree of association (monomers per polymer), M represents the monomer molecular weight, $\bar{v}$ is the protein partial specific volume (0.74 ml/g), ρ is the solution density (assumed to be unity), f is the polymer frictional coefficient, and N is Avogadro's number. The frictional coefficient for a sphere of equivalent volume, f_e, is given by Stokes law

$$f_e = 6\pi\eta r_e \tag{3}$$

where η is the solvent viscosity and r_e is the radius of the equivalent sphere calculated from

$$\frac{4}{3}\pi r_e^3 = \frac{nM}{N}(G + \bar{v}) \tag{4}$$

Combination of eq 2–4 gives

$$\frac{f}{f_e} = \left(\frac{nM}{\pi N}\right)^{2/3}\frac{(1 - \bar{v}\rho)}{6\eta s}\left[\frac{3}{4}(G + \bar{v})\right]^{-1/3} \tag{5}$$

and f/f_e is related to axial ratio of the hydrodynamic unit (polymer) through Perrin's equation (Perrin, 1936). Consequently, eq 1 relates hydrodynamic unit axial ratio to viscosity and eq 5 relates it to sedimentation. For the addition of salt ($I = 2$) to β-casein and unit weight ratio mixtures of α_s- and β-caseins, the viscosity and sedimentation data are in good and closest agreement when the polymer axial ratio is unity. It seems likely, therefore, that these polymers are spherical in shape or at least very nearly so.

For spheres, the required quantities G, n, and the solvated radius, r, are obtained from

$$G = \frac{[\eta]}{2.5} - \bar{v} \tag{6}$$

$$n = \frac{N\pi}{M}\left[\frac{3}{4}\left(\frac{6\eta s}{1 - \bar{v}\rho}\right)^3(G + \bar{v})\right]^{1/2} \tag{7}$$

$$r = \frac{nM(1 - \bar{v}\rho)}{N \quad 6\pi\eta s} \tag{8}$$

These equations give, for the β-casein polymer, a solvation of 2.2 g of H_2O/g of protein, a degree of polymerization of 31, giving a molecular weight of 747,000, and a radius for the polymer of 95 Å. These results are summarized in Table II and evidently are in agreement with the degree of polymerization and polymer radius obtained by Archibald analysis.

The unit weight ratio α_s–β-casein polymer is calculated to have a solvation of 3.8 g of H_2O/g of protein, 28 monomer units/polymer, a polymer weight of 720,000, and a radius of about 108 Å. Again there is good agreement with the data obtained from Archibald analysis (Table II). The β- and α_s–β-casein polymers appear to be much alike. This result is not unexpected in light of the extensive interactions which occur and implies that the α_s-casein and β-casein monomer units must be similar in size, shape, and the manner of charge distribution.

The data so far examined provide the first of a number of comparisons which suggest that α_s- and β-caseins comingle at the molecular level in the development of polymers. The experimental results to be compared are that β-casein precipitates at $I = 4.5$, 1:1 α_s–β-caseins at $I = 3.5$ and α_s-casein at $I = 0.5$ when total protein concentration is 10 mg/ml. A comingling of α_s- and β-caseins has also been suggested by Ashworth (1964) and Payens (1968). If hydrophobic interaction is retained as the main source of polymer–polymer interaction causing the formation of precipitates in the absence of divalent cation, it appears that β-casein is less active in this respect than α_s-casein. Possibly the conformations of the acidic peptides affect the extent to which body–body hydrophobic interactions can occur between polymers.

In this investigation the solvation of ammonium sulfate precipitates of α_s-casein is found to be near 1.6 g of water/g of protein. This value is close to the minimum solvation of both Ca–α_s-caseinate precipitates ($G = 1.6$, Waugh and Noble, 1965) and the micelles of milk ($G = 1.9$). If a comparison is made of polymer and precipitate solvations it is evident that polymer spheres must overlap in the production of precipitate. The extent of maximum overlap, assuming undistorted spherical polymers, is readily calculated to be about 30%. It is significant that if charge is reduced by reducing pH, solvation is quite different; the isoionic precipitate of α_s-casein has $G = 0.79$.

The development of polymers, to the point of precipitate formation, has been examined for α_s- and β-caseins and unit weight ratio mixtures using calcium as the driving ion. In

these studies either calcium concentration was held constant and temperature increased (Figure 2 shows viscosity data, Figure 5 sedimentation data) or temperature was held constant and calcium concentration increased (Figure 4 shows sedimentation data).

β-Casein at 0.015 M Ca, $I = 0.048$, has significant plateau regions in $s_{20,w}$ and $[\eta]$ between 21 and 25° while precipitation takes place between 26 and 27°. From the sedimentation and viscosity data we obtain, for an axial ratio near unity, $G = 4.4$, $n = 30$, and $r = 114$ Å. These are in agreement with data obtained by Archibald analysis (Table II).

In other cases, and particularly when α_s-casein is present, polymer development follows a more complex route and the complete polymer does not appear to occur as the major structure in solution just before precipitation. This is most evident with α_s-casein alone where, with increasing calcium concentration (Figure 4), an initial set of polymers increases in $s_{20,w}$ but at 0.003 M Ca, a fast shoulder appears on the slower distribution and thereafter as Ca increases two distributions are observed. The area of the fast peak increases and that of the slow peak decreases until, just above 0.007 M Ca, a precipitate forms. If $s_{20,w} = 19.5$ S and $r = 105$ Å be taken for the limiting spherical polymer, the approximate degree of association is 33 and $G = 2.6$.

The unit weight ratio α_s–β-casein system may be used to illustrate further points. At $I = 0.048$ and 0.009 M Ca, there appear to be plateaus, near 23°, for both $s_{20,w}$ (Figure 5) and $[\eta]$ (Figure 2). These systems have higher $[\eta]$ and lower $s_{20,w}$ than systems in the plateau region at high ionic strength. If spherical polymers are assumed and plateau values used, $G = 5.1$, $n = 12$, and $r = 88$ Å. The polymers appear to be incomplete and the small plateaus to represent temperature ranges over which polymer properties alter slowly. It is suspected that, with increasing temperature, the processes of polymer completion and surface interaction occur simultaneously to develop the precipitate. However, a comingling at the polymer level is again indicated by comparison of the behavior of β-casein, the unit weight ratio mixture and α_s-casein at $I = 0.048$, 0.009 M Ca, and 23°. α_s-Casein alone precipitates while the others remain in solution. Further, at $I = 0.048$, 0.015 M Ca, and 23°, α_s-casein precipitates while the mixture and β-casein remain in solution.

That near-spherical polymers of appropriate size are incorporated directly into the micelle core is suggested by electron micrographs of micelles of milk. These indicate that the micelles have crenated surfaces and are built up of subunits which leave "open" spaces in the micelle core. These open spaces accumulate stain. The subunits are thought to be roughly spherical in shape and about 100 Å in diameter in the dried state (Shimmin and Hill, 1964, 1965).

A precipitate of core polymers having the structure proposed here should have a random monomer orientation over distances of a few core polymer diameters. Available evidence indicates that this is the case. Tuckey *et al.* (1938) found that appropriate X-ray diffraction spacings, which would indicate preferred orientation, are absent and in addition, we have found that precipitates are optically isotropic.

The monomer structures proposed above should also account reasonably for the viscosities given by solutions of α_s- and β-caseins when the proteins are present in monomeric form. Ho and Chen (1967) have obtained intrinsic viscosities, $[\eta]$, for α_s-casein in 0.01 M KCl and pH 7. The intrinsic viscosity of α_s-casein is 10.2 ml/g at 37°. Sullivan *et al.* (1955) have obtained the intrinsic viscosity of monomeric β-casein in Veronal buffer at pH 7.78, 7.6°, and ionic strength 0.1 and have obtained a value of 20.2 ml/g. This is close to the value of 23.3 ml/g obtained by Noelken and Reibstein (1968) using 0.04 M NaCl–0.02 M EDTA, pH 7, and 2.5° ($I = 0.1$). Clearly, α_s- and β-caseins are not typical globular proteins since the latter have intrinsic viscosities in the range of 3.3–4.0. (Tanford, 1961). Our purpose now is to show for each protein, that a body length can be chosen which accounts for radii of complete polymers and that reasonable acidic peptide characteristics can then be introduced to account for viscosities of monomer solutions; that is, the acidic peptides entrap solvent and by this means make a contribution to the properties of the hydrodynamic unit: to solvation, axial ratio and, at low ionic strengths, the electroviscous effect. It should be recognized that lack of information, mainly concerning polymer surface detail (polymers are probably not smooth spheres), gives some latitude in the choice of body length.

α_s-Casein was examined by Ho and Chen at low ionic strength and an electroviscous contribution would be expected. The latter can be estimated according to Booth (1951) as contributing about one-third of the measured intrinsic viscosity at $I = 0.01$. The intrinsic viscosity of the hydrodynamic unit is then 6.7 and this is related to the viscosity increment, ν^*, by eq 1, thus

$$\nu^*(0.74 + G) = 6.7 = [\eta] \tag{1}$$

where G is now the total amount of solvent, g of water per g of protein, associated with the monomer hydrodynamic unit, as an ellipsoid, by Simha's equation (Simha, 1940; Mehl *et al.*, 1940).

The solvation, G, can be divided into two parts: body solvation and loop solvation. Since the acidic peptide accounts for essentially all of the net charge on the molecule, the body is expected to be nearly isoionic and to bind solvent to an extent comparable with that of the protein in an isoionic precipitate. The total measured solvation of isoionic precipitates of α_s-casein is 0.79 g of water/g of protein. The isoionic precipitate is assumed to contain closely packed ellipsoids consisting of protein molecules including bound water. The ellipsoids will then occupy ~0.74 of the total volume and the remainder will be free space in which solvent is occluded. Then

$$0.74 = \frac{G_b + \bar{v}\rho}{G_I + \bar{v}\rho} \tag{9}$$

where G_b is the body solvation (bound solvent), G_I is the total solvation of the isoionic precipitate (0.79), and ρ is the solvent density, 1 g/ml. Using these values gives $G_b = 0.4$ g of water/g of protein, a value in the customary range obtained from studies of protein hydration.

From the monomer in solution the total solvation, G, is then given by

$$G = \frac{G_b M_b + N(V_a - V_p)\rho}{M_b + M_a} \tag{10}$$

where V_a represents the volume of the acidic peptide region, V_p is the volume of the acidic peptide protein, and M_b and M_a are the molecular weights of the body and acidic peptide, respectively.

At low ionic strength and pH 7, the acidic peptide loop of α_s-casein could be a ring of length about 145 Å, calculated from peptide molecular weight of ~5000. This yields a radius for the loop of ~23 Å, a hydrated radius of ~25.5 Å, and width of ~17 Å. Placing the loop midway between body center and end (see Figure 6), where the radius of the ellipsoid body is ~10 Å, gives a volume dominated by the loop of ~29,400 Å.^{3} From eq 3, G is calculated to be 0.84 and eq 1 gives $\nu^* = 4.25$. Assuming that this hydrodynamic unit can be approximated by an ellipsoid permits use of Simha's equation, in which case $\nu^* = 4.25$ corresponds to a hydrodynamic unit axial ratio of 3.6. The length of the body and hydrodynamic unit having been equated, volume equations for ellipsoids give a body length of 110 Å and diameter of 22 Å. This length is reasonably close to the radius of the limiting core polymer. The approximations used make body length insensitive to the positioning of the loop. The length is also found to be relatively insensitive to the volume dominated by the loop.

It is possible that the electroviscous correction may be too large. As the electroviscous correction for α_s-casein is reduced, the intrinsic viscosity increases and, by eq 1, this requires an increase in the volume dominated by the loop and/or an increase in hydrodynamic unit axial ratio. Both can be obtained for constant body length by moving the loop toward the end of the body. For example, a value of $[\eta] = 10.2$ (no correction) can be accounted for by placing the loop as an extension to the body such that it has an effective length of 50 Å and dominates a volume of 24,600 Å^3. G is then 0.73 by eq 10 and ν^* is 6.9 by eq 1. ν^* by Simha's equation gives an axial ratio of 4.8 for the hydrodynamic unit. The body length remains at 110 Å. Ho and Chen (1967) found that α_s-casein has an intrinsic viscosity of 19.2 ml/g in 6 M guanidine hydrochloride (Gd·HCl). From the viscosity increase over that in 0.01 M KCl they conclude that the molecule of α_s-casein does not ordinarily exist as a completely random coil.

For β-casein (Sullivan *et al.*, 1955), $[\eta] = 20.2$ at 7.6°. Inasmuch as this was measured at $I = 0.1$, there should be no necessary correction for the electroviscous effect. This high intrinsic viscosity suggests that the acidic peptide is attached near the end of the body and is extended in such a way as to markedly increase hydrodynamic unit axial ratio. This peptide chain, which has a length of ~87 Å, could readily dominate a volume of ~22,000 Å^3. The axial ratio of the hydrodynamic unit would then be ~9.6 and the axial ratio of the body ~6.5. The body length would be ~13 Å and its diameter ~20 Å. This body length, by comparison with polymer radii given in Table II, appears to be too great. It would be reduced to a more acceptable value if there were an additional three to five amino acid residues in the solvent accessible peptide.

Extraction of traces of calcium by EDTA could cause conformational changes in the acidic peptide of β-casein which would account for the higher value of $[\eta] = 23.3$ obtained by Noelken and Reibstein (1968) as compared with $[\eta] = 20.2$ obtained by Sullivan *et al.* (1955). Noelken and Reibstein observed no increase in viscosity in going from dilute salt to 6 M Gd·HCl. They interpreted this to indicate that β-casein ordinarily exists in solution as a random coil. However, according to the proposed model, formation of a random coil in Gd·HCl would decrease hydrodynamic unit axial ratio and the contribution to the viscosity from the acidic peptide but increase the contribution from the body. The net effect might be small.

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