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Physical Parameters of κ-Casein from Cow's Milk*

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Received April 17, 1964; revised July 27, 1964

The association of κ-casein molecules into aggregates in inorganic salt solutions near neutrality precludes a study of the basic unit with these conditions. Previous studies on κ-casein were performed in alkaline phosphate buffers (≈*p*H 12) to avoid molecular aggregation. However, the properties of κ-casein treated in this manner indicate a destruction of the indigenous disulfide bonds. Consequently, the physical parameters of the protein were determined in neutral or acid solvents containing agents capable of dissociating secondary bonds. The weight-average molecular weight of κ-casein, determined by the short-column sedimentation-equilibrium procedure, was approximately 125,000 in 67% acetic acid–0.15 M NaCl and 5.0 M guanidine HCl solutions. A value of approximately 56,000 for the small component was determined from Trautman plots of approach-to-equilibrium data for the polydispersed system in 7.0 M urea, 33% acetic acid–0.15 M NaCl, and, at low protein concentrations, in 5.0 M guanidine HCl. A molecular weight of approximately 28,000 was obtained for a disulfide-reduced sample of κ-casein in both 5.0 M guanidine HCl and 67% acetic acid–0.15 M NaCl solutions. The reduced specimen was monodispersed in these solvents. The molecular weight of κ-casein in alkaline phosphate buffer (*p*H 12.0) was approximately 28,000; but chemical analyses indicated a loss of disulfide bonds. The results of these studies suggest that κ-casein is composed of two subunits of approximately 28,000 mw joined by disulfide bonds or by secondary bonding which is dependent on specific tertiary configuration produced by intramolecular disulfide bonds. Values for the minimum molecular weight of κ-casein calculated from phosphorus, sulfur, and sialic acid analyses support this conclusion.

κ-Casein is believed to function as the "protective colloid" for micellar casein. The interaction of this protein with other casein components in the presence of calcium ions at concentrations where certain of the components are individually insoluble yields a stable micelle (Waugh and Von Hippel, 1956; Waugh, 1958, 1961). The alteration of κ-casein by the enzyme rennin is sufficient to cause coagulation of the micellar proteins (Waugh, 1958; Wake, 1959; Garnier, 1959). Reflecting its capability for strong interactions, κ-casein forms relatively uniform aggregates of high molecular weight in inorganic salt solutions below *p*H 11 (Waugh and Von Hippel, 1956). These aggregates must be dissociated to characterize more precisely the physical parameters of this protein. However dissociation can be accomplished only by using strong agents, e.g., concd acetic acid, guanidine HCl, or urea, which make the determination of thermodynamic or hydrodynamic properties less accurate because of the preferential interactions and the nonideal behavior of the protein. Recent studies of various proteins in dissociating agents similar to those employed here showed that these errors were not greatly significant (Harrap and Woods, 1961; Kielley and Harrington, 1960; Trautman and Crampton, 1959; Yphantis and Waugh, 1957; Criddle *et al.*, 1962).

In this paper we report: (a) some of the physical properties of κ-casein in concentrated acetic acid, guanidine HCl, and urea, (b) a partial compositional analysis of the protein, and (c) data to support the conjecture that the basic unit of κ-casein is composed of two subunits joined by disulfide bonds. Selected portions of this study were reported previously without detail by Swaisgood and Brunner (1963).

* Supported in part by a grant (EF 247) from the National Institutes of Health. Published with the approval of the Michigan Agricultural Experiment Station as Journal article No. 3361.

† Presently located at the Eastern Utilization Research and Development Division, USDA, Philadelphia. This investigation was carried out during the tenure of a predoctoral fellowship from the Division of General Medical Sciences, U.S. Public Health Service.

EXPERIMENTAL

Preparation of κ-Casein.—The protein was located from freshly drawn cow's milk by a trichloroacetic acid–urea extraction procedure described previously by Swaisgood and Brunner (1962). The preparation was further purified by centrifugation in a solution of 0.1 M NaCl in the 50-L rotor (Spinco) at 50,000 rpm for 6 hours at 4°. The pellet and bottom one-third layer in the centrifuge tube were collected, dispersed, and passed through a Sephadex G-75 column previously equilibrated with 0.1 M NaCl. The purified κ-casein was collected in the eluate immediately following the void volume.

Chemicals.—*p*-Mercuribenzoate was obtained from the Sigma Chemical Co. Glutathione (GSH)[1] was supplied by the Matheson Co. Guanidine HCl and 2-mercaptoethanol were Eastman products. The guanidine HCl was recrystallized from a 1:1 (v/v) mixture of absolute methanol and ethyl ether as described by Greenstein and Jenrette (1942). The urea, obtained from Mallinckrodt, was recrystallized from 60% ethanol and dried at <60° in a vacuum desiccator.

Composition Analyses.—The amino acid composition of the κ-casein preparation was determined with a Beckman Amino Acid Analyzer on 24-, 48-, and 72-hour acid hydrolysates. The values reported were corrected to zero time for the amino acids which decreased in concentration with time of hydrolysis. The quantity of protein analyzed was determined from a micro-Kjeldahl analysis for nitrogen.

Sialic acid was measured colorimetrically by the thiobarbituric acid method reported by Aminoff (1961). Sialic acid was released from the protein by hydrolysis with 0.1 N H_2SO_4 at 80° for 1 hour. *N*-Acetylneuraminic acid obtained from General Biochemicals was employed as a standard.

Phosphorus was determined colorimetrically in a wet digest (H_2SO_4 and H_2O_2) of the protein by the method of Sumner (1944). Ferrous sulfate was added in slight excess to reduce the phosphomolybdic acid to a blue color, which was quantitatively measured at 610 mμ. Total sulfur was determined by the Spang Microanalytical Laboratory, Ann Arbor, Mich. The protein was dried over P_2O_5 to constant weight in a vacuum oven at 105°.

Electrophoresis.—Zonal-electrophoretic analyses were performed in urea-starch gels according to the method of Wake and Baldwin (1961). A discontinuous buffer system consisting of Tris-citrate buffer incorporated into the gel and borate buffer in the buffer compartments was employed. The gel was made 7 M with respect to urea. Electrophoresis was performed at approximately 2 mA/cm (gel width) in a cold room (≈5°) for approximately 16 hours.

Ultracentrifugation.—Sedimentation-velocity and sedimentation-equilibrium studies were performed in a Spinco Model E analytical ultracentrifuge equipped with an RTIC temperature-control unit and a phase plate as a schlieren diaphragm. A capillary-type synthetic-boundary cell was used in most of the velocity experiments for the following reasons: (a) the prevalent sedimentation coefficients were <1.0 S; (b) to eliminate the problem of restricted diffusion at the meniscus; and (c) because concentrated urea and guanidine HCl solutions form gradients at the air meniscus in the conventional analytical cell, making observations of the protein boundary difficult. Equilibrium studies were performed in the double-sector cell. Centerpieces were of the filled-Epon type. A false bottom of FC-43 fluorocarbon oil was employed in the short-column equilibrium experiments. The photographic plates were analyzed with a Nikon microcomparator capable of measuring to <0.002 mm.

The sedimentation-velocity studies were performed at 25° with rotor speeds of 59,780 rpm. Sedimentation coefficients, determined by plotting the logarithm of the maximum ordinate against time, were corrected to values corresponding to water at 20° ($s_{20,w}$). The concentration of protein corresponding to a particular $s_{20,w}$ represented the average concentration in the first and the last frame used to calculate the sedimentation coefficients and was corrected for radial dilution according to Schachman (1959).

The data obtained from the short-column equilibrium experiments were analyzed according to Van Holde and Baldwin (1958). The weight-average ($\overline{M}_w$) and *z*-average ($\overline{M}_z$) molecular weights for the entire cell contents were computed from the following expressions:

$$\overline{M}_w = \frac{2RT}{(1 - \bar{\nu}\rho)\omega^2(x_b^2 - x_m^2)} \cdot \frac{c_b - c_m}{c_o}$$

and

$$\overline{M}_z = \frac{RT}{(1 - \bar{\nu}\rho)\omega^2} \cdot \frac{1/x_b \left(\frac{dc}{dx}\right)_b - 1/x_m \left(\frac{dc}{dx}\right)_m}{c_b - c_m}$$

In these equations R represents the gas constant, T the absolute temperature, c the protein concentration determined by measuring the area under the refractive index gradient curve, ω the angular velocity, $\bar{\nu}$ the partial specific volume, ρ the solution density, and x the distance in cm from the axis of rotation (where the subscripts b and m refer to the bottom and the top of the liquid column, respectively).

The molecular weight of the small component in polydispersed systems was evaluated according to Trautman's (1956) treatment of approach-to-equilibrium data obtained at various speeds and as adapted to heterogeneous systems by Erlander and Foster (1959). Thus the experimental data were plotted according to the equation

$$\left(\frac{dc}{dx}\right)_m / \omega^2 x_m = \frac{-M(1 - \bar{\nu}\rho)}{RT} [(C_o - C_m) - C_o]$$

and the molecular weight for the small component was calculated from the slope of the plot at rotor speeds where only the small component left the meniscus. Diffusion coefficients were calculated from the patterns obtained during the short-column equilibrium experiments according to the method of Sophianopoulos *et al.* (1962).

The basic data for estimating the protein concentration were established by dissolving a predetermined amount of dry protein directly into the buffer. The volume was carefully checked before and after dialysis and the concentration was confirmed by analysis for nitrogen. For the purpose of molecular-weight calculations, the area under the schlieren curve formed in a synthetic-boundary cell was used as a measure of the protein concentration.

Density and Partial Specific Volume.—Solvent densities were determined with 25-ml pycnometers equilibrated in a temperature-controlled water bath at 25.0 ± 0.01°. The partial specific volume of κ-casein was calculated from its amino acid composition, as reported herein, and its carbohydrate content, as reported by Jollès *et al.* (1962). Solution densities were calculated according to Fujita (1962), using values for the solvent density and the partial specific volume of the solute.

Viscosities Measurements.—Relative viscosities were determined in a Cannon-Ubbelohde semimicrodilution

[1] Abbreviation used in this work: GSH, glutathione.

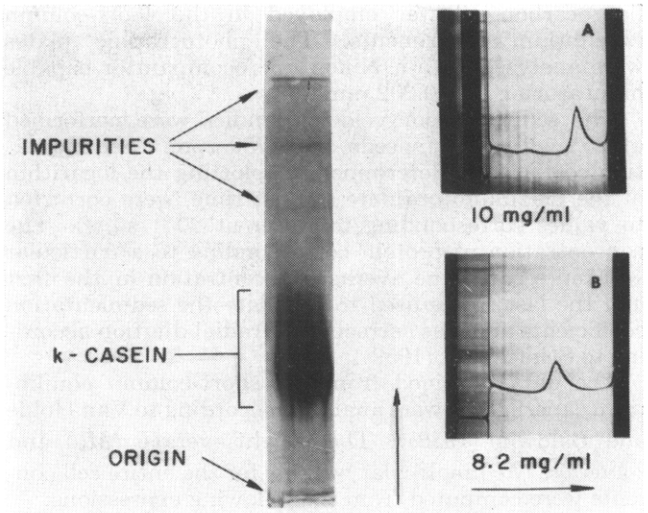

FIG. 1.—Starch-gel (7 M urea) electrophoresis and sedimentation-velocity patterns of κ-casein. Sedimentation studies were performed in phosphate buffer, *p*H 7.0, Γ/2 = 0.1 at 25° for ≈20 minutes: pattern A, synthetic-boundary cell; pattern B, standard analytical cell.

viscometer at 25.0 ± 0.01°. The outflow time for water was 285.8 seconds. Four to six observations of the outflow time were obtained for each determination. The intrinsic-viscosity values were established by extrapolating to zero protein concentration a plot of reduced viscosity (η_{sp}/c) versus *protein* concentration (g/ml).

Reduction of Disulfide Bonds and SH-Group Titration.—The disulfide bonds of κ-casein were reduced with mercaptoethanol at a level of *ca.* 2 μl mercaptoethanol/mg of protein in 5 M guanidine HCl adjusted to *p*H 8.4 with methylamine (Anfinsen and Haber, 1961). Following a reduction period of 24 hours the solution was adjusted to *p*H 3 with glacial acetic acid. The reducing agent (i.e., mercaptoethanol and guanidine HCl) were removed by passing the solution over Sephadex G-25 equilibrated with 0.1 M acetic acid. Complete elution of the protein was achieved approximately 40–50 ml prior to the elution of the mercaptoethanol.

Sulfhydryl groups were titrated essentially according to the procedure described by Boyer (1954). However, to prevent reoxidation of the reduced disulfide bonds following the removal of mercaptoethanol, the following procedure was devised: (a) The protein concentration was determined on an aliquot of the protein solution immediately following its elution in 0.1 M acetic acid from the Sephadex G-25 column; (b) excess *p*-mercuribenzoate was added directly to the reduced protein in 0.1 M acetic acid; and (c) the *p*H was adjusted to 7 with 0.1 N NaOH. The detailed procedure is as follows: (1) The volume of protein solution was accurately measured after removing the aliquot and before adding the *p*-mercuribenzoate solution. The amount of protein in the reaction mixture was usually 15–20 mg. (2) A known volume of standard *p*-mercuribenzoate solution containing approximately ten times the quantity of *p*-mercuribenzoate required to react with the newly formed SH groups was added to the reduced κ-casein. The concentration of the standard *p*-mercuribenzoate solution was determined by titration with a freshly prepared standard GSH solution (Benesch and Benesch, 1962). This method provided results which were in good agreement with the concentration of GSH calculated from the extinction coefficient at 232 mμ as described by Boyer (1954). (3) The *p*-mercuribenzoate-containing protein solution was quantitatively transferred to a 100-ml volumetric flask. After standing for 1 hour at *p*H 7, the solution was made 7 M with respect to urea to insure complete reaction of the SH groups, and diluted to volume. The unreacted *p*-mercuribenzoate in aliquots of this solution was titrated with the standard GSH solution previously used to titrate the standard *p*-mercuribenzoate solution. Thus standard GSH solution was added to both the sample and reference cells in equal volumes with a Starrett microsyringe, and the end point was determined by following the change in absorbancy at 250 mμ. A protein-free blank consisting of a volume of 0.1 M acetic acid was treated similarly. The difference between the amount of *p*-mercuribenzoate in the blank and the sample was presumed to represent the quantity of *p*-mercuribenzoate bound to the protein.

RESULTS

Purity of the κ-Casein Preparation.—As shown in Figure 1, the starch-gel electrophoretic pattern displayed the typical κ-casein smear just ahead of the starting slot (Wake and Baldwin, 1961; Neelin *et al.*, 1962). A small amount of residual protein contamination was indicated by the presence of faint leading bands. Likewise, the sedimentation-velocity diagrams (Fig. 1) for κ-casein run in a synthetic-boundary cell (A) in phosphate buffer (*p*H 7.0; Γ/2 = 0.2) showed a small amount of slow-sedimenting component which could not be detected in the regular analytical cell (B). Similar results were obtained for a McKenzie-Wake (1961) preparation of κ-casein. Free-boundary electrophoresis of our preparation in buffers ranging from *p*H 6.0 to 8.6, Γ/2 = 0.1, showed but one boundary. The κ-casein preparation was estimated to be about 97–99% homogeneous.

TABLE I
THE AMINO ACID COMPOSITION OF κ-CASEIN

Amino Acid	Grams/100 g[a]	W_i[b]	Residues/28,000 g: This Study[c]	Jollès *et al.*[d]	α_3-Casein[e]
Asp	7.72	6.68	16	15	16
Thr	6.74[f]	5.72	16	16	10
Ser	5.03[f]	4.17	13	16	15
Glu	19.80	17.38	38	33	34
Pro	10.95	9.24	27	21	25
Gly	1.23	0.93	5	5	5
Ala	5.40	4.31	17	17	17
Cys				1.6	1.6
Val	6.30	5.33	15	12	12
Met	1.68	1.48	3	2	2
Ileu	7.10	6.13	15	13	14
Leu	6.11	5.27	13	13	14
Tyr	7.61	6.86	12	11	15
Phe	3.86	3.44	7	7	7
Lys	6.51	5.71	12	11	13
His	2.36	2.09	4	3	3
Arg	3.96	3.55	6	6	7
Try				1	2
NH_3	1.94[f]	1.82	32		35
	$\sum_i W_i$[g] = 95.12				

[a] Based on a nitrogen content of 15.3%. [b] Weight percentage of the *i*th amino acid residue. [c] Analysis provided through the courtesy of Drs. J. P. Riehm, J. S. Speck, and H. A. Lillevik of the biochemistry department. [d] Calculated from the g/100 g protein reported by Jollès *et al.* (1962). [e] Calculated from the g/100 g protein reported by Hipp *et al.* (1961) for α_3-casein. [f] Extrapolated values. [g] This summation includes, in addition to the amino acid residues, the values for sialic acid (1.4%, $\bar{\nu}$ = 0.59) and phosphorus (based on the H_2PO_3 residue) as well as the values for Cys, Try, galactose (1.4%, $\bar{\nu}$ = 0.62), and galactosamine (1.2%, $\bar{\nu}$ = 0.62) reported by Jollès *et al.* (1962).

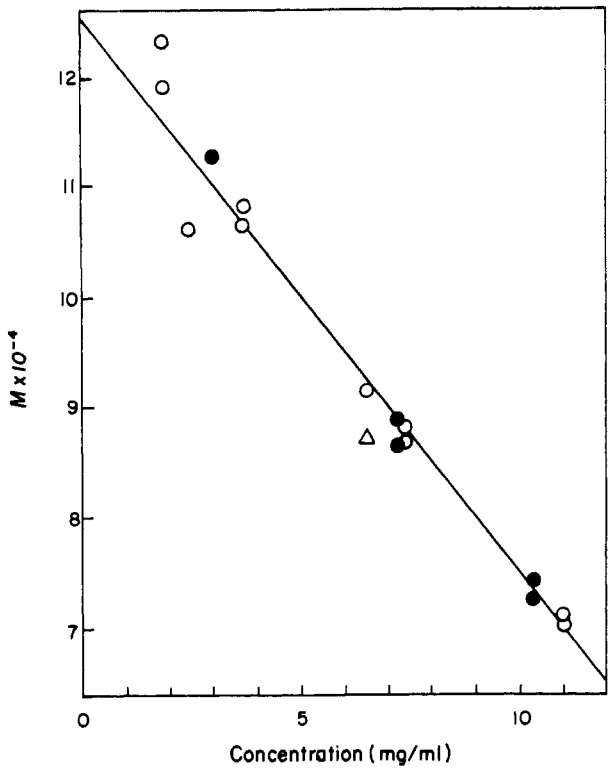

FIG. 2.—Concentration dependency of the apparent molecular weight of κ-casein in dissociating solvents. Legend: O, M_w in 67% acetic acid–0.15 M NaCl; ●, M_w in 5 M guanidine HCl, pH 4.8–4.9; △, M_w of Waugh and Von Hippel's purified fraction S in 67% acetic acid–0.15 M NaCl.

TABLE II
MOLECULAR WEIGHTS OF κ-CASEIN CALCULATED FROM COMPONENT ANALYSES

Component	Concentration (%)	Minimum Molecular Weight	Number of Residues	Molecular Weight
Sialic acid (NAN)	1.4	22,000	1	22,000
Phosphorus	0.22	14,090	2	28,180
Sulfur	0.70	4,570	6	27,420
Tryptophan[a]	1.05	19,500	1	19,500

[a] Reported by Jollès *et al.* (1962).

Composition of κ-Casein.—The amino acid composition of κ-casein is shown in Table I. These values are based on a nitrogen content of 15.3%. The weight percentages of the amino acid residues are listed together with a comparison of the number of amino acid residues per 28,000 g of protein for (a) our preparation; (b) the κ-casein studied by Jollès *et al.* (1962), which was obtained by the method of McKenzie and Wake (1961); and (c) the α_3-casein reported by Hipp *et al.* (1961a). A summation of the residue weights for our preparation, which includes in addition to the amino acid residues the values for sialic acid and phosphorus as well as the values reported by Jollès *et al.* (1962) for cystine, tryptophan, galactose, and galactosamine, accounted for approximately 95% of the protein. From these data and the specific volumes of the residues a value of 0.73 was calculated for the partial specific volume of κ-casein.

The concentrations of sialic acid, tryptophan, phosphorus, and sulfur, together with the corresponding calculated minimum molecular weights of κ-casein, are shown in Table II. Jollès *et al.* (1962) and Thompson and Pepper (1962) reported phosphorus concentrations of 0.217 amd 0.22%, respectively. Waugh (1958) reported a value of 0.19% for a preparation which appeared to be approximately 90% pure. The reported values for sialic acid show more variation, seemingly dependent upon the procedure used to prepare the specimen (Marier *et al.*, 1963).

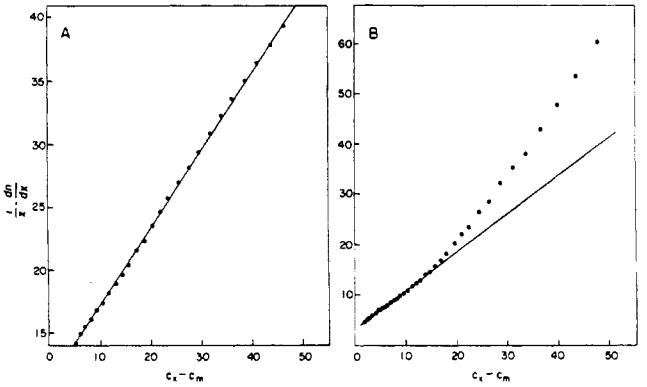

FIG. 3.—Van Holde-Baldwin plots of equilibrium data for κ-casein run at 12,590 rpm and 25°. A, 7.34 mg/ml of protein in 67% acetic acid–0.15 M NaCl; B, 3.0 mg/ml of protein in 5.0 M guanidine HCl, pH 4.8–4.9.

Physical Properties of κ-Casein.—The short-column-equilibrium technique was used to determine the molecular weight of κ-casein at various concentrations in 5.0 M guanidine HCl and 67% acetic acid–0.15 M NaCl. The results of these studies are tabulated in Table III and plotted in Figure 2. A marked concentration dependency was noted in these two solvents. An extrapolation to zero protein concentration yielded a molecular weight in the order of 125,000. These values were not corrected for charge effects or preferential interactions. However the charge effect should not be large at the ionic strengths employed and the error due to preferential interactions should not be great in concentrated acetic acid (Schachman, 1960), 6.0 M guanidine HCl (Kielley and Harrington, 1960), or concentrated urea (Trautman and Crampton, 1959). Furthermore, the values for the molecular weights obtained in various solvents during the course of this study were similar.

The $\overline{M}_z/\overline{M}_w$ ratio was approximately 1 in 67% acetic acid–0.15 M NaCl but approached a value of 2 at a protein concentration of 3 mg/ml in 5.0 M guanidine HCl. Shown in Figure 3, the Van Holde-Baldwin plot was interpreted to indicate molecular polydispersity at low concentrations of protein in 5.0 M guanidine HCl, whereas a nearly linear relationship was observed in 67% acetic acid–0.15 M NaCl at all concentrations of protein. A single determination in 7.0 M urea at 5 mg/ml gave a $\overline{M}_w$ = 108,000 and a $\overline{M}_z/\overline{M}_w$ of nearly 2.

Approach-to-equilibrium experiments were performed at various rotor speeds to determine the molecular weight of the small component in these polydisperse systems. The data were analyzed from a Trautman plot as discussed by Erlander and Foster (1959); see Figure 4. From these measurements the molecular weight of the small component was estimated to be 54,000, 58,000, and 57,000 in 5.0 M guanidine HCl, 33% acetic acid–0.15 M NaCl, and 7 M urea, respectively. A straight line, indicative of molecular homogeneity, was obtained for κ-casein in 67% acetic acid–0.15 M NaCl.

Hydrodynamic properties of κ-casein are given in Table IV. The sedimentation-velocity data for the protein in 67% acetic acid–0.15 M NaCl fit the linear relation $s_{20,w} = 2.7\ (1 - 0.0302c)$ and, in 6 M guanidine HCl, $s_{20,w} = 3.18\ (1 - 0.0178c)$, where $s_{20,w}$ is in Svedbergs and c in mg/ml. A combination of these $s_{20,w}$

TABLE III
SEDIMENTATION-EQUILIBRIUM MOLECULAR-WEIGHT DATA FOR κ-CASEIN AND MERCAPTOETHANOL-REDUCED κ-CASEIN

κ-Casein			Mercaptoethanol-reduced κ-Casein		
c_p (mg/ml)	$M_w \times 10^{-4}$	$\bar{M}_z/\bar{M}_w$	c_p (mg/ml)	$M_w \times 10^{-4}$	$\bar{M}_z/\bar{M}_w$
Solvent: 67% acetic acid–0.15 M NaCl					
0	12.42		0	2.73	
1.83	12.09	1.11	3.2	2.65	0.99
2.40	10.60[a]		6.3	2.55	0.91
3.67	10.74	1.17	9.6	2.48	1.13
6.50	9.14	1.01			
6.50	8.71[a,b]	1.14[a,b]			
7.34	8.75	0.98			
11.0	7.03	0.91			
Solvent: 5.0 M guanidine HCl, pH 4.8					
0	12.61		0	2.87	
3.0	11.28	1.94	3.0	2.77[a,c]	
5.5	9.44	1.18[a]	6.0	2.52[a,c]	
7.2	8.73	1.18	9.0	2.49[a,c]	
10.3	7.38	1.17	7.0	2.1[a,b,d]	

[a] Value for one calculation. [b] Value for fraction *S* (Waugh and Von Hippel, 1956) purified by ultracentrifugation and elution from Sephadex G-75. [c] Value for the *p*-mercuribenzoate derivative of mercaptoethanol-reduced κ-casein. [d] Solvent was 5.0 M guanidine HCl, pH 8.4, containing 7 μl/ml of mercaptoethanol.

TABLE IV
HYDRODYNAMIC PROPERTIES OF κ-CASEIN AND MERCAPTOETHANOL-REDUCED κ-CASEIN

Property	κ-Casein			Mercaptoethanol-reduced κ-Casein		
	Phosphate Buffer, pH 7.0	67% Acetic Acid–0.15 M NaCl	5.0 M Guanidine HCl	Phosphate Buffer, pH 12.2	67% Acetic Acid–0.15 M NaCl	5.0 M Guanidine HCl
$s^\circ_{20,w} \times 10^{13}$	15.6	2.7	3.18	1.4[a]	1.26	1.88
k[b]	0.0165	0.0302	0.0178	0.0172	0.0146	0.0106
$D_{20,w} \times 10^7$		2.4 (c = 0)	3.1 (10.3 mg/ml)	5.8 (8 mg/ml)[a]	5.7 (6.4 mg/ml)	5.8 (9 mg/ml)
$[\eta]$ (ml/g)	9.5	35	31	15.1		
f/f_0		3.2	2.7	2.3	2.6	1.8
$\beta \times 10^{-6}$		1.8	2.0	2.04		
M $\times 10^{-4}$	M_s = 65	$M_{s,D}$ = 10.1	$M_{s,D}$ = 9.3[c]	$M_{s,D}$ = 2.23[c]	$M_{s,D}$ = 2.0[c]	$M_{s,D}$ = 2.0[c]

[a] Swaisgood and Brunner (1962). [b] From the equation $s^\circ_{20,w}(1 - kc)$. [c] Neglecting the concentration dependency of the diffusion coefficient.

values and the equilibrium molecular weights determined in the same solvents revealed axial ratios of approximately 3. Diffusion coefficients were 2.4 ficks at zero concentration in 67% acetic acid–0.15 M NaCl and 3.1 ficks in 5.0 M guanidine HCl at a protein concentration of 10.3 mg/ml. Insertion of these data into the Svedberg equation yielded values for the molecular weight which were slightly lower than values determined from the equilibrium experiments. The intrinsic viscosity of κ-casein in 0.1 M NaCl at a neutral pH was 9.5 ml/g. Dissolving the protein in 67% acetic acid–0.15 M NaCl or 5.0 M guanidine HCl resulted in a rise in the intrinsic viscosity to 35 ml/g and 31 ml/g, respectively (Fig. 5). These data were used to compute the values listed in Table IV for the Scheraga-Mandelkern (1953) constant.

Physical Properties of Reduced κ-Casein.—The apparent molecular weights for mercaptoethanol-reduced κ-casein at various concentrations in 67% acetic acid and 5 M guanidine HCl are shown in Table III. For these experiments, the protein was reduced in 5.0 M guanidine HCl at pH 8.4. A portion of this solution was adjusted to pH 3.0 with acetic acid and dialyzed against 67% acid–0.15 M NaCl solvent. The remaining portion of the reduced protein was converted to its *p*-mercuribenzoate derivative for analysis in the guanidine HCl solvent. The reoxidation of SH groups should be minimized under these conditions. The molecular weights thus obtained for the mercaptoethanol-reduced κ-casein were not as concentration dependent as those of the nonreduced protein (see Fig. 6). Van Holde-Baldwin plots indicated that molecular monodispersity existed at all observed protein concentrations. The $\bar{M}_z/\bar{M}_w$ ratio was approximately 1 in 67% acetic acid. However, in 5.0 M guanidine HCl, the $\bar{M}_w$ could not be determined throughout the entire cell because of the difficulty experienced in the formation of a completely symmetrical synthetic boundary. Molecular weights at zero protein concentration in 67% acetic acid and 5 M guanidine HCl were 27,300 and 28,700, respectively.

Selected hydrodynamic parameters for the mercaptoethanol-reduced κ-casein are compared with the nonreduced protein in Table IV. The sedimentation coefficients for the reduced form in 67% acetic acid were best represented by the relation $s_{20,w}$ = 1.26 (1–0.146 c). A value of 2.6 for f/f_0 was calculated from the sedimentation data and the average equilibrium molecular weight (i.e., 28,000). The diffusion coefficients were 5.7 and 5.8 ficks in 67% acetic acid and 5.0 M guanidine HCl, respectively.

Determination of Sulfhydryl Groups.—The nitroprusside test for free-sulfhydryl groups in 5.0 M guanidine HCl gave negative results for our preparation and a κ-casein[2] prepared by the method of Waugh and Von Hippel (1956). Following a treatment with mercaptoethanol, the test was positive. The *p*-mercuribenzoate experiments showed the presence of 2–3 SH groups per 28,000 g of κ-casein.

Analyses for total sulfur and methionine showed, by

[2] Fraction *S*, additionally purified by the procedure outlined in this paper.

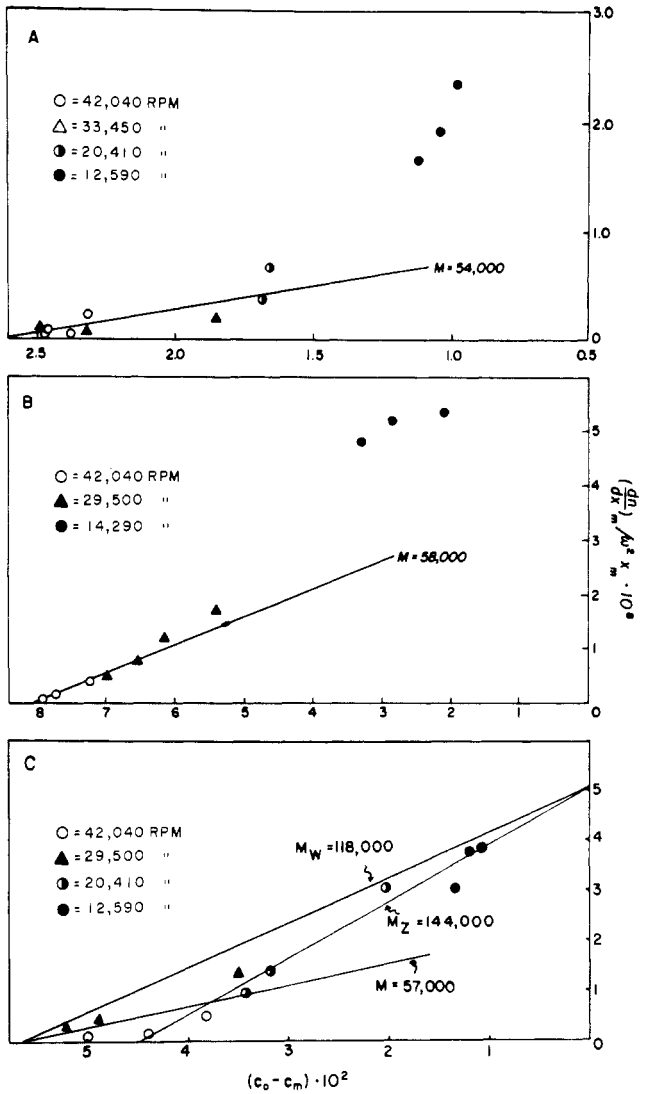

FIG. 4.—Trautman plots of approach-to-equilibrium data for κ-casein in various dissociating agents. A, in 5.0 M urea, *p*H 4.8–4.9; B, in 33% acetic acid–0.15 M NaCl; C, in 7.0 M urea, *p*H 8.5.

difference, three half-cystine per 28,000 mw. These data are summarized in Table V.

Effect of High pH.—κ-Casein was dissolved in *p*H 12.2 phosphate buffer, $\Gamma/2 = 0.19$, and allowed to stand for 48 hours at room temperature prior to reduction of the disulfide bonds with mercaptoethanol. Treated in this manner the protein showed a negative nitroprusside test and did not bind *p*-mercuribenzoate (Table V). Furthermore, κ-casein dissolved in *p*H 12.2 phosphate buffer (5.3 mg/ml) exhibited a molecular weight of 23,400 as determined from a linear Trautman plot of approach-to-equilibrium data; and when dialyzed into 5.0 M guanidine HCl and analyzed by equilibrium sedimentation it showed a molecular weight of 24,500. These results agree favorably with the molecular weight value of 26,000 reported by McKenzie and Wake (1959) for κ-casein in *p*H 12 phosphate buffer.

A comparison of the hydrodynamic properties of alkaline-treated κ-casein with those of the mercaptoethanol-reduced specimen indicates that both treatments engender similar physical dimensions in the protein.

DISCUSSION

Chemical Properties.—A comparison of the amino acid composition of the κ-casein used in this study with that

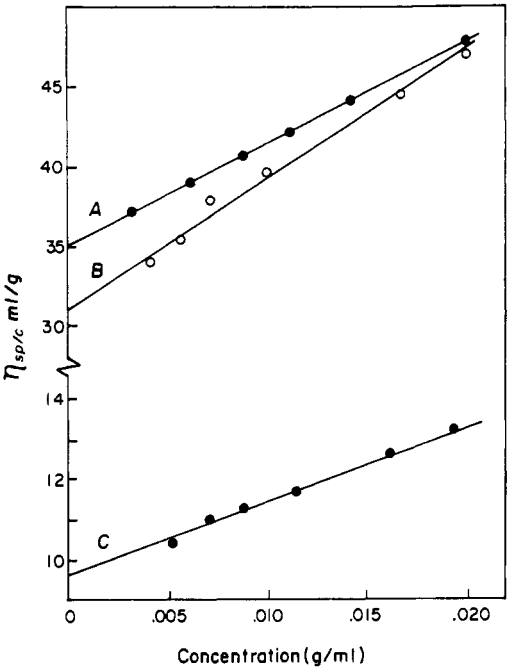

FIG. 5.—Reduced viscosity for κ-casein as a function of concentration. Line A, in 67% acetic acid–0.15 M NaCl; line B, in 5.0 M guanidine HCl, *p*H 4.8–4.9; line C, in 0.1 M NaCl.

TABLE V
SULFHYDRYL GROUP DETERMINATIONS IN κ-CASEIN

Protein	Method of SH-Group Analysis	Number SH/ 28,000 g	Nitroprusside
Mercaptoethanol-reduced κ-casein	Titration of unreacted *p*-mercuribenzoate with GSH		Positive
	Trial 1 (18.9 mg)[a]	1.93	
	Trial 2 (15.5 mg)[a]	2.67	
	Direct determination of mercaptide at *p*H 9.5	1.82	
κ-Casein	Calculated from sulfur from methionine composition	2.98	Negative
κ-Casein prepared by the method of Waugh and Von Hippel (1956)			Negative
κ-Casein adjusted to *p*H 12 followed by reduction	Titration of unreacted *p*-mercuribenzoate with GSH	0.1	Negative

[a] Total amount of protein reacted with excess *p*-mercuribenzoate.

of the preparation studied by Jollès *et al.* (1962) showed good agreement and the values were similar to those reported for α_3-casein by Hipp *et al.* (1961a). Like κ-casein, α_3-casein was stable to calcium ion, a substrate for the primary action of rennin and stabilized α_s-casein in the presence of calcium ions (Hipp *et al.*, 1961b). But α_3-casein was higher in phosphorus (0.35%), less soluble at neutral *p*H, and possessed a higher s_{20} in phosphate buffer at *p*H 7 (i.e., 23 S as compared to 13–15 S for κ-casein).

The concentration of sialic acid reported for our preparation lies in the range of values previously reported for κ-casein. Marier *et al.* (1963) found 2.14%

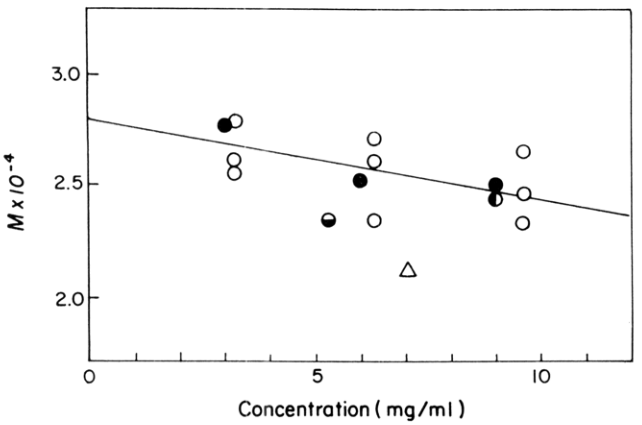

FIG. 6.—Concentration dependency of the molecular weight of reduced κ-casein in dissociating solvents. Legend: ○, M_w for reduced κ-casein in 67% acetic acid–0.15 M NaCl; ●, M_w for *p*-mercuribenzoate—κ-casein in 5 M guanidine HCl, *p*H 4.8–4.9; △, M_w for Waugh and Von Hippel's fraction *S* in 5 M guanidine HCl, *p*H 8.4 and containing 7 μl/ml of mercaptoethanol; ◒, *M* from Trautman plot of approach-to equilibrium data for κ-casein in phosphate buffer, *p*H 12.2, Γ/2 = 0.12; ◐, M_w for κ-casein treated at *p*H 12.2, then dialyzed against 5 M guanidine HCl, *p*H 4.8–4.9.

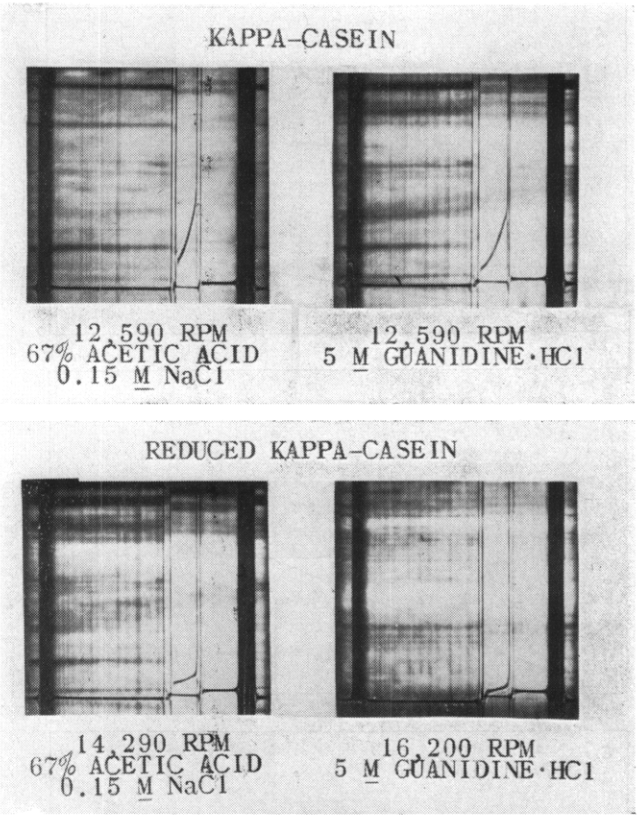

FIG. 7.—Sedimentation-equilibrium patterns for κ-casein and mercaptoethanol-reduced κ-casein in 67% acetic acid–0.15 M NaCl and 5.0 M guanidine HCl at 25°. Concentration of protein was ≈6.5 mg/ml.

sialic acid in a specimen obtained by the Swaisgood-Brunner (1962) method. The specimen was not purified as described herein and appeared to contain more of the leading bands when assayed by starch-gel electrophoresis. Possibly some of the contaminating proteins contain sialic acid as suggested by the observations of Malpress (1961) that only 68% of the sialic acid in whole casein was released by rennin, whereas Marier *et al.* (1963) found that approximately 90% of the sialic acid was released from κ-casein.

Physical Properties.—Molecular weights of approximately 125,000 were obtained for κ-casein from sedimentation-equilibrium data in 67% acetic acid–0.15 M NaCl and 5.0 M guanidine HCl. The protein was essentially monodispersed in 67% acetic acid–0.15 M NaCl. At low protein concentrations in 5.0 M guanidine HCl, 7.0 M urea, and in 0.33% acetic acid–0.15 M NaCl, the system exhibited gross polydispersion. The $\overline{M}_w$ was similar to that obtained in 67% acetic acid–0.15 M NaCl, however the $\overline{M}_z$ was nearly doubled. Determinations for the molecuar weight of the small component in three solvents (i.e., 33% acetic acid–0.15 M NaCl, 5.0 M guanidine HCl, and 7.0 M urea,) gave an average value of 56,000. These observations suggest that κ-casein displays conditional polymorphic interactions. For example, by lowering the concentration of acetic acid or the concentration of prote n in 5.0 M guanidine HCl a monomer-polymer equilibrium was favored over that of the single species observed in 67% acetic acid–0.15 M NaCl. The molecular weight reported for the basic unit (i.e., 56,000) is in agreement with values proposed by Beeby (1963) and Garnier *et al.* (1962) which were based on a release of glycomacropeptide and protons, respectively, by the action of rennin.

An unusual interaction of κ-casein monomers to give polymers of nearly equal size occurs in neutral salt solutions. The size is independent of temperature, ionic strength, *p*H, or the presence of calcium ions (Waugh, 1958). Thus sedimentation-velocity data obtained from various experiments (using different preparations and including a specimen prepared according to McKenzie and Wake [1961]) performed at temperatures of 3–25° and ionic strengths of 0.1–0.5 were found to follow the linear relation $s_{20} = 15.6\ (1 - 0.0165c) \pm 0.25$. A rough approximation of the molecular weight of this aggregate can be obtained from the Scheraga-Mandelkern (1953) equation since β is relatively insensitive to particle shape. According to the equation given by Schachman (1959), a molecular weight of roughly 650,000 was calculated.

Cleavage of the disulfide bonds with mercaptoethanol resulted in a decrease in the molecular weight of κ-casein in 5.0 M guanidine HCl and 67% acetic acid–0.15 M NaCl. This reduction in the molecular weight is readily apparent in the schlieren curves, shown in Figure 7, which also indicates a high degree of molecular homogeneity. The Van Holde-Baldwin (1958) plots for reduced κ-casein were also linear, as expected, since the ratio $\overline{M}_z/\overline{M}_w$ was nearly equal to 1. An average molecular weight of approximately 28,000 was obtained for the reduced protein by extrapolating the data to zero protein concentration. The decrease in molecular weight was also evidenced by the increase in the diffusion coefficients and decrease in the sedimentation coefficients. These results do not seem to be a consequence of the method of preparation since κ-casein prepared by the methods of Waugh and Von Hippel (1956), which do not employ either concentrated urea or trichloroacetic acid, behaved similarly. Further support for a subunit size of 28,000 was provided by the molecular weights estimated from the chemical composition, i.e., approximately 24,300.

These experimental observations suggest: (a) that the basic unit of κ-casein is composed of two 28,000-mw subunits joined by disulfide bonds, or (b) that the two subunits are joined by secondary bonds which depend on the disulfide bonds to maintain a particular tertiary structure for the interaction. The first possibility seems more tenable and is supported by the cystine content of κ-casein. Three half-cystines per 28,000 g were indicated by the sulfur and methionine contents

and by the cystine values of the preparations studied by Jollès *et al.* (1962) and Hipp *et al.* (1961a). Also, 2–3 SH groups per 28,000 g of reduced κ-casein reacted with *p*-mercuribenzoate. The fact that whole casein does not contain cysteine was first reported by Kassel and Brand (1938) and confirmed for κ-casein by the nitroprusside tests conducted in this study. If the cystines are evenly distributed between the two subunits an odd number per subunit weight would preclude at least one intermolecular disulfide bond. Further, if only one glycomacropeptide is released per 50,000 g of κ-casein, as suggested by the data of Beeby (1963) and Garnier *et al.* (1962), the two subunits should be different. Studies are in progress to separate and characterize the individual chains. But their sizes must be nearly similar since the sedimentation-equilibrium data for reduced κ-casein were characteristic of a single molecular species.

At high values of *p*H, the disulfide bonds of κ-casein undergo a cleavage. The physical properties of the protein in phosphate buffer at *p*H 12.2 were similar to those of the reduced protein in 67% acetic acid and 5 M guanidine; and the alkaline-treated protein possessed the same molecular weight in 5 M guanidine HCl as did the reduced form. The exposure of κ-casein to a high-alkaline environment followed by the addition of mercaptoethanol yielded a reduced form which gave a negative nitroprusside test and failed to bind *p*-mercuribenzoate. These results are not surprising considering the lability of disulfide bonds to alkali (Cecil and McPhee, 1959). For example, Brown *et al.* (1959) detected an odor of hydrogen sulfide in neutralized aqueous solutions of ribonuclease previously exposed to *p*H 12.7 for 30 minutes at room temperature. More recently, Young and Potts (1963) observed a 70% loss of half-cystine in ribonuclease after 2 hours at *p*H 11 in 5 M guanidine HCl.

Although the hydrodynamic data are not precise enough for making calculations of particle dimensions, e.g., accurate corrections for charge effects and preferential interactions are not available, an approximation of the particle shape was determined. The high frictional ratios (f/f_0) calculated for κ-casein in 67% acetic acid–0.15 M NaCl, 5.0 M guanidine HCl, and *p*H 12.2 phosphate buffer indicated that the molecules were either quite asymmetric or possessed an unusually large degree of solvation. A calculation of the Scheraga-Mandelkern (1953) constant gave values of 1.8×10^6, 2.0×10^6, and 2.04×10^6. The theoretical minimum value for β of a spherical molecule is 2.12×10^6. The values reported here are probably within the experimental error of this minimum. Consequently, we suggest that the molecules underwent an isotropic swelling rather than a linear extension in the polypeptide chains as a result of exposure to dissociating solvents. Similar conclusions were drawn by Scheraga and Mandelkern (1953) for the case of horse serum albumin in concentrated urea solutions. The frictional ratios they observed indicated axial ratios of about 20:1, whereas the calculated β values ranged from 1.98 to 20.5×10^6. Also, Harrap and Woods (1961) found that the intrinsic viscosity of bovine albumin was greatly increased when dissolved in anhydrous formic acid. However, on the basis of light-scattering data, they concluded that isotropic swelling had occurred rather than extension of the peptide chains.

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