THE ISOLATION AND PHYSICAL-CHEMICAL CHARACTERIZATION OF KAPPA-CASEIN FROM COW'S MILK

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ABSTRACT

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The casein micelle of milk represents a complex of many interacting proteins, one of which - kappa-casein - stabilizes the other members in their ionic environment. However, the strong interactions between the micellar proteins impedes the isolation and characterization of the individual constituents. The purpose of this study was to develop a procedure for the isolation of kappa-casein and to investigate its chemical and physical properties.

These studies employed sedimentation-velocity and sedimentation-equilibrium ultracentrifugation extensively, as well as electrphoresis, viscosity, and various chemical analytical techniques.

A procedure was developed for obtaining good yields of crude kappa-casein of about 90% purity. The crude kappa-casein was readily purified to approximately 97% purity for physical-chemical studies. The characteristics of the preparation were found to be similar to those previously reported for kappa-casein.

Association of kappa-casein molecules to form large aggregates of relatively uniform size in inorganic salt solutions near neutrality precludes the study of the basic unit in these buffers. Therefore, the physical properties of kappa-casein were determined in strong dissociating solvents. The weight-average molecular weight of the protein was approximately 125,000 in 67% acetic acid - 0.15 M NaCl and 5.0 M guanidine·HCl solutions. A molecular weight of approximately 56,000

was obtained for the low-molecular weight component in 7.0 M urea, 33% acetic acid - 0.15 M NaCl, and at low protein concentrations in 5.0 M guanidine·HCl.

Reduction of the disulfide bonds caused the molecular weight to be lowered to 28,000 in 5.0 M guanidine·HCl and 67% acetic acid - 0.15 M NaCl. Physical-chemical studies showed that the disulfide bonds were also destroyed in phosphate buffer at pH 12.

Determination of the sulfhydryl groups by several chemical methods indicated 2-3 -SH groups per 28,000 g. An odd number of -SH groups per 28,000 g suggested at least one inter-molecular disulfide bond since there were no free -SH groups. Minimum molecular weights calculated from the chemical analyses supported the physical studies.

These data led to the conclusion that the basic unit of kappa-casein was composed of two 28,000 molecular weight sub-units joined by disulfide bond(s).

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INTRODUCTION

The casein micelle of milk is a complex system of interacting proteins and calcium ions. The micelles are in equilibrium with polymer complexes of the individual components, the equilibrium being displaced towards the micellar form by calcium ion addition. Nevertheless, the system is remarkably stable to rigorous industrial processing treatments such as pasteurization, sterilization, concentration, drying, reconstituting, freezing, and addition of sugar and/or inorganic salts. However, lowering the pH to 4.6 releases the calcium ions and the casein fraction precipitates.

At least four individual proteins are present in the micelle, two of which (α_s- and β-casein) are individually insoluble at calcium ion concentrations normal to milk. There are undoubtedly more than four constituents since the λ-casein fraction appears heterogeneous. The principle micellar system appears to be a complex of α_s- and k-casein.

The interactions leading initially to complexes and finally to micelles are strong and highly specific. This behavior was responsible for earlier conclusions that casein was a homogeneous substance. For example, the α-casein fraction appears to be homogeneous by free-boundary electrophoresis and ultracentrifugation and, in fact, can be isolated intact from 4.6 M urea solutions. However, studies involving the addition of calcium ion or strong dissociating solvents show that α-casein is a complex of k-, α_s- and probably the λ-casein fraction. Furthermore, these strong interactions make the isolation and purification of any one of the components difficult. Consequently the information concerning the properties of the individual components is incomplete.

k-Casein has been shown to be the "protective colloid" of the micelle system. The interaction of this protein with the other components yields a stable micelle and in its absence the other caseins (with the exception of λ-casein) will precipitate at low calcium ion concentrations. Also, the alteration of this single protein by the primary action of the enzyme rennin is sufficient to cause coagulation of the micellar proteins. Moreover, the interaction seems to be stoichiometric since a weight-ratio of 4 α_s-: 1 k-casein is preferred for complex formation. Relatively pure k-casein forms polymers of nearly uniform size in buffers not considered to be strong dissociating agents. In fact, this protein seems to interact with itself and other proteins more readily than any of the other micellar components. The polymers formed are relatively independent of pH, temperature, ionic strength, and calcium ion concentration suggesting that this is a rather unusual specific interaction.

In view of the above discussion, k-casein would appear to present a challenging system for the study of secondary bonding forces in proteins. However, before such a study can be pursued three objectives must be achieved: a) a method for preparing considerable quantities of pure protein must be developed, b) the chemical composition must be determined, and c) elementary physical properties such as molecular weight, sedimentation coefficient, diffusion coefficient, intrinsic viscosity, and shape must be described. The purpose of the research described herein was to achieve, insofar as possible, these three objectives.

For the sake of organization, the thesis has been divided into two parts: Part I dealing with the method of preparation and preliminary characterization of k-casein and Part II describing the physical-chemical studies performed on the preparation.

HISTORICAL

General

Linderstrøm-Lang (1925) first recognized the calcium-insensitivity of a casein fraction which he called Z-casein. Later he (Linderstrøm-Lang, 1929) proposed the existence of a "protective colloid" in the casein fraction which was responsible for the stabilization of the casein micelle and was specifically attacked by rennin. The classical electrophoretic separation of whole casein into α-, β- and γ-casein, in order of decreasing mobility, was reported by Mellander (1939). Subsequently, Warner (1944) suggested that α-casein[1] was heterogeneous and later, Nitschmann and Lehmann (1947) proposed that rennin did not act directly on α-casein, but rather, on a component thereof.

The "protective colloid" theory has been the subject of considerable controversy. Recent experiments have supported this hypothesis so that at present it is universally accepted. The factor most responsible for the renewed impetus in casein research was the discovery of kappa-casein by Waugh and von Hippel (1956). While attempting to purify micellar casein, they obtained a fractionation of the casein into calcium-sensitive and calcium-insensitive fractions. Analysis of the calcium-insensitive fraction (Fraction S) revealed the presence of β-casein and a new component which they called k-casein. Furthermore, addition of Fraction S to the calcium-sensitive fraction in the correct proportions resulted in the formation of stable micelles in the presence of calcium ion.

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[1] This component has now been definitely shown to be heterogeneous and will be referred to as the α-casein fraction (Brunner, Ernstrom, Hollis, Larson, Whitney, and Zittle, 1960).

Methods Employed in the Preparation of k-Casein

Method of Waugh and von Hippel (1956)

This method essentially consists of a physical separation at constant pH. Micelles were collected from skimmilk by adjusting the calcium ion concentration to 0.06 M causing nearly all of the casein to concentrate in the micellar form, and centrifuging at 45,000 X G. Calcium ion was removed from the precipitate -- designated as first-cycle casein -- by using a chelating agent, e.g. sodium oxalate or citrate. First-cycle casein was made 0.25 M with respect to $CaCl_2$ and the calcium-sensitive fraction removed by centrifugation, first at 900 X G (37° C) and then at 90,000 X G (5° C). The supernatant, designated as Fraction S, contained about 70% k-casein. Later, Waugh (1958) reported a purification of Fraction S using centrifugation, salting-out, and isoelectric precipitation which yielded k-casein of approximately 90% purity. The contaminate consisted largely of m-casein which is probably similar to the λ-casein described by Long (1958), see Brunner et al. (1960).

Method of Long (1958)

The starting material for this procedure was the α-casein fraction prepared by the method of Warner (1944). A 2 - 3% protein solution was adjusted to 0.2 M $CaCl_2$ concentration and the calcium-sensitive fraction removed by centrifuging initially at 35° C and 9,000 RPM and finally at 25° C and 21,000 RPM. The k-casein obtained was contaminated with a slow-sedimenting component as shown by sedimentation-velocity patterns. The slow-sedimenting component was obtained by collecting the top layer from crude k-casein solutions centrifuged for 5 hours at 40,000 RPM.

The protein recovered was shown to be different from previously described caseins and was designated as λ-casein. It was stable to calcium ion, possessed an S_{20} of ca 1.3 S at pH 7, contained 1.18% phosphorus, and did not stabilize α_s-casein or give a visible reaction with rennin.

Method of Fox (1958) as Modified by Morr (1959)

k-Casein was prepared from whole casein or from Warner's α-casein fraction. The preparative operations were performed at 0 - 5° C unless otherwise stated. The starting material was adjusted to pH 11.3 and held for 20 - 30 min. Calcium chloride was added to a final concentration of 0.2 M, the pH lowered to 8.3 and the resulting precipitate removed. The temperature was raised to 30° C causing more calcium-sensitive material to precipitate. The supernatant was adjusted to pH 4.7, dialyzed against water, and the precipitate collected. These steps were repeated to further remove the calcium-sensitive fraction. The precipitate was dissolved in 6.6 M urea and crude k-casein obtained by diluting to 3.3 M urea concentration. Crude k-casein was again dissolved in 6.6 M urea and the pH adjusted to 11.3 for a short time. After lowering the pH to 4.7 and diluting to 3.3 M urea concentration, a brown precipitate was removed and k-casein was precipitated from the supernatant by salting-out (1.5 M ammonium sulfate). These steps were repeated twice. The k-casein obtained appeared homogeneous by sedimentation-velocity studies.

Method of Pilson, Henneberry, and Baker (1960) for Fraction A

α-Casein fraction prepared by the method of Warner was adjusted to pH 12 at 25° C for 45 min, then lowered to pH 7.0, followed by addition of $CaCl_2$ to 0.25 M concentration. Calcium sensitive α-casein (α_s-casein)

was removed by centrifugation and Fraction A was precipitated by adjusting the pH to 4.5. The precipitate was redissolved and the steps repeated. The material obtained was homogeneous by paper electrophoresis. The preparation was considered similar to Fraction S on the basis of the stability to calcium ion, reaction with rennin, and ability to stabilize α_s-casein.

Method of McKenzie and Wake (1961)

Whole acid casein was treated with $CaCl_2$ (ca 0.4 M) at pH 6.5 - 7.0. The calcium-sensitive fraction was removed by warming to 35° C and centrifuging at room temperature, first at 900 X G followed by 40,000 X G. After removing the calcium ion with oxalate, the supernatant was warmed to 25° C and 25g/100ml of anhydrous Na_2SO_4 was added. The precipitate (k- and β-casein) was removed by centrifugation (900 X G), dissolved in water and dialyzed against 0.005 M NaCl. Following adjustment of the pH to 7.2, an equal volume of absolute ethanol was added, followed by 2 M ammonium acetate until a definite mucilaginous precipitate formed. The precipitate was dissolved in 6 M urea and dialyzed against 0.005 M NaCl. If the dissolved precipitate was dialyzed against water or too long against 0.005 M NaCl, the k-casein precipitated and was only soluble with difficulty. The preceding steps with ethanol and ammonium acetate were repeated to assure complete removal of β-casein. Residual fat was removed from the final product by centrifuging at 90,000 X G. k-Casein was obtained in yields of ca 20% of theory and appeared homogeneous by velocity-ultracentrifugation and urea-starch gel electrophoresis.

Method of Cheeseman (1962)

α-Casein fraction prepared by the urea method of Hipp, Groves, Custer and McKeekin (1952) was treated with oxalate to remove residual calcium. The solution was made 3.3 M with respect to urea at 4° C and the pH adjusted to 4.9, followed by dilution to 2 M urea concentration which precipitated α_s-casein. The supernatant, containing k-casein, was dialyzed against water and adjusted to 0.2 M $CaCl_2$ concentration at pH 6.6 - 7.0. Calcium-sensitive material was removed by centrifugation at 37° C and 2500 RPM. The preparation was 90% pure by free-boundary electrophoresis at pH 7.3. About 25% recovery was obtained assuming 15% k-casein in whole casein.

Method of Hill (1963)

Whole acid casein was made 0.25 M with respect to $CaCl_2$ at 3° C and pH 6.7 - 7.2. After removing the calcium-sensitive fraction by warming to 35° C and centrifuging at 5000 RPM, the supernatant was dialyzed against water and concentrated. The solution was again treated with $CaCl_2$, but this time centrifuged at 50,000 X G. The crude k-casein was applied to a DEAE cellulose column in pH 6.25 acetate buffer. A gradient elution was obtained by mixing the previous buffer with various amounts of pH 4.5 acetate buffer adjusted to 0.5 M with respect to $CaCl_2$. Under these conditions, β-casein was eluted first, whereas k-casein was not eluted until the $CaCl_2$ molarity had increased and the pH was lowered. k-Casein was soluble at pH 4.7 in 0.5 M $CaCl_2$. The purity of the preparation was not determined by the common physical methods.

Method of Hipp, Groves, and McMeekin (1961) for Preparing α_3-Casein

Although the protein was not designated as such, it is undoubtedly similar, if not identical, to k-casein and therefore will be described here. The calcium-insensitive casein was prepared from Warner's α-casein fraction by centrifuging a solution of the protein adjusted to 0.2 M $CaCl_2$ concentration at 3500 X G at room temperature. Further purification of the calcium-insensitive protein was accomplished by refrigerated centrifugation at 40,000 RPM for 5 hours in 0.2 M NaCl. The pellet obtained was recentrifuged twice under the same conditions, except in the absence of salt.

α_3-Casein was homogeneous by urea-starch gel electrophoresis, free-boundary electrophoresis at pH 2.3 (μ = 3.6 Tiselius units), and velocity-ultracentrifugation at pH 7 (S_{20} = 23.1 S). The protein appeared to have less stabilizing power than k-casein, however, it was coagulated by rennin yielding 17% of the total nitrogen as soluble nitrogen. It was soluble to the extent of only 0.26% at pH 6.9. The sialic acid content was 1.2% and phosphorus 0.35%.

Chemical Properties of k-Casein

Composition

Amino acids. The amino acid composition obtained on a sample prepared by the McKenzie-Wake method was reported by Jollès, Alais, and Jollès (1962). The results are presented later in the thesis for comparison with an analysis obtained on k-casein prepared by the procedure developed in this laboratory. The absence of cysteine in the casein fraction was first reported by Kassel and Brand (1938). Jollès et al.

(1962) compared their analysis to that of Hipp, Basch, and Gordon (1961) for α_3-casein. k-Casein appeared to have a higher content of the hydroxy-amino acids than α_3-casein.

Nitrogen. Reported values for the nitrogen content have varied, possibly due to the extent of purity of the various preparations. Cheeseman (1962) reported 13.6%, Beeby (1963) 14.6%, Hipp et al. (1961) 14.6% for α_3-casein, and Pilson et al. (1960) 13.9% for Fraction A.

Phosphorus and NANA. Similarly various values have been reported for the phosphorus and N-acetylneuraminic acid (NANA) composition. Phosphorus contents reported in the literature are 0.33% (Long, 1958), 0.19% for purified Fraction S (Waugh, 1958), 0.22% (Thompson and Pepper, 1962) and 0.217% (Alais and Jollès, 1961) for preparations by the McKenzie-Wake method, and 0.92% (Pilson et al., 1960) for Fraction A. The percentages of NANA for the various preparations were reported as 0.79% (Cheeseman, 1962); 1.2 - 2.0% (Hill, 1963); 2.5%, 1.8%, and 2.4% for different McKenzie-Wake preparations (Thompson and Pepper, 1962; Beeby, 1963; and Alais and Jollès, 1961 respectively); 2.14% for a preparation obtained by the method described in this thesis (Marier, Tessier, and Rose, 1963); and 2.22% for Fraction A (Cayen, Henneberry, and Baker, 1962). Some of the variation was undoubtedly due to the various analytical procedures employed. Alais and Jollès (1961) have shown that N-acetylneuraminic acid is the only sialic acid present in bovine k-casein. According to the latter authors, k-casein also contains 1.2% galactosamine and 1.4% galactose.

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Stabilization of α_s-Casein

At calcium ion concentrations normal to milk, α_s-casein by itself is insoluble (Waugh and von Hippel, 1956; Waugh, 1958). However, addition of increasing proportions of k-casein caused an increase in soluble α_s-casein. When the weight-ratio of α_s-/k-casein was 4 : 1, all of the α_s-casein was present in a stable micelle at 0.03 M $CaCl_2$ concentration and 37° C (Waugh and von Hippel, 1956). Once formed, the micelle was stable even at 0° C. However, when the micelle formation experiment was performed at 0° C, the α_s-casein was not stabilized by k-casein at a weight-ratio of 4 unless whey proteins were present (Waugh, 1958 and 1961). They observed the same weight-ratio to be the naturally preferred ratio in the absence of calcium at pH 7, or if the complex was formed from monomers of the components by short exposure to pH 12. Free k-casein was observed in the sedimentation-velocity pattern in the case of lower ratios at pH 7. This stoichiometry led Waugh (1958) to propose a molecular model for the complex containing three molecules of α_s-casein and one of k-casein.

At the higher temperatures, the complex incorporated β-casein. β-Casein was also observed to interact with k-casein polymers as demonstrated by the fact that Fraction S did not become cloudy at 37° C in the presence of calcium ion (normally concentrations of β-casein similar to that in Fraction S would become cloudy). The function of m-casein (similar to Long's λ-casein and Hipp's α_2-casein) in the micelle and its interactions with k-casein have not been determined (Waugh, 1958 and 1961; Long, 1958).

Zittle (1960 and 1961) described a test for determination of the stabilizing power of k-casein. Using his particular preparation of k-

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casein, Zittle (1960) found that 80% of the α_s-casein was stabilized when the ratio of k-/α_s-casein was 0.1 and the test solution contained 10 mg α_s/ml. The stabilization was less at lower concentrations of α_s-casein. Later, 90% stabilization at a ratio of 0.1 was reported using a McKenzie-Wake preparation (Zittle, 1961). Release of NANA by neuraminidase decreased somewhat the stabilizing power of k-casein (Thompson and Pepper, 1962).

Reaction with Rennin

Alais, Mocquot, Nitschmann, and Zahler (1953) demonstrated an increase in 12% TCA-soluble nitrogen (NPN) resulting from the action of rennin on casein. With the discovery of k-casein and the demonstration of its ability to stabilize the casein micelle, Waugh and von Hippel (1956) proposed that this protein was the primary substrate for rennin action. Subsequent studies indicated that fractions rich in k-casein liberated more NPN when serving as rennin substrates (Garnier, 1957 and 1959; Wake, 1957 and 1959). For example, Wake (1959) found a 6.7% increase in NPN for k-casein, but only 1% increase when first-cycle casein was studied. From these data, he calculated that k-casein comprised 15% of the whole casein, a result which agreed with the physical studies of Waugh and von Hippel (1956).

Waugh (1958) reported that Fraction S prepared from rennin treated skimmilk contained m-casein but not k-casein. However, the calcium-sensitive fraction (Fraction P) behaved similarly to that obtained from untreated milk. Furthermore, the time required to convert k-casein into para-k-casein was comparable to the time required to clot skimmilk, whereas α_s-casein and β-casein were not altered in this interval.

Immunoelectrophoretic studies have shown four components in the α-casein fraction, only one of which was altered by the action of rennin (Garnier, 1959 and 1960). Also, urea-starch gel electrophoresis of whole casein has revealed many bands, but only the k-casein band was altered by rennin treatment (Wake and Baldwin, 1961).

The peptide released from whole casein by rennin and soluble in 12% TCA was shown to be non-dialyzable and essentially electrophoretically homogeneous (Alais, 1956). Later, Nitschmann, Wissmann, and Henzi (1957) showed that this material was a glyco-macropeptide (GMP) containing galactose, glucosamine, and sialic acid but no free alpha amino groups and that its molecular weight by sedimentation-diffusion ranged between 6000 and 8000. The carbohydrate content of GMP from whole casein was verified (Brunner and Thompson, 1959).

Since all previous data indicated that k-casein was the primary substrate for rennin, large proportions of GMP having the same composition as that obtained from whole casein should be released from this protein. Indeed, several workers have shown that the GMP obtained from whole casein, α-casein fraction, and k-casein (prepared by the McKenzie-Wake method) was the same (Nitschmann and Beeby, 1960; Jollès and Alais, 1960; Alais and Jollès, 1961; Jollès, Alais, and Jollès, 1961). Starting with k-casein, Nitschmann and Beeby observed an 8% increase in NPN and obtained a GMP containing 11.2% N, 0.5% P, and 60% of the total GMP in the peptide portion. This GMP had the same amino acid composition as that obtained from whole casein or from the α-casein fraction. Confirming amino acid analyses were reported by Jollès and co-workers (1960 and 1961). Using their data, Jollès *et al.* (1960 and 1961) calculated the molecular weight of GMP to be 8000 ± 500. This preparation contained 0.4% P, 10.1% N,

28.2% carbohydrate, and 72% of the total weight in the peptide portion. Galactosamine, galactose, and sialic acid represented 24%, 26%, and 50% respectively of the total carbohydrate moiety of GMP, the same as in the original k-casein.

The GMP contained 74% of the carbohydrates in the original k-casein, whereas GMP from whole casein accounted for only 31% of the total carbohydrate (Alais and Jollès, 1961). These data suggest that not all of the carbohydrate in whole casein resides in the k-casein molecule. Malpress (1961) found that only 68% of the sialic acid in whole casein was released by rennin. Alais and Jollès (1961) indicated that further investigations are necessary to determine whether or not all of the carbohydrate of k-casein is located in the GMP. Wake (1959) suggested that some of the GMP is precipitated in 12% TCA. However, Beeby (1963) stated that all of the sialic acid was soluble in 12% TCA and therefore the GMP must also be completely soluble. The possibility that peptides other than the GMP are released must also be considered (Nitschmann and Henzi, 1959).

Jollès *et al.* (1961) could find no N-terminal amino acids in the GMP, confirming Nitschmann's earlier work, however, two yellow DNP derivatives were observed and the possibility was suggested that these may be derivatives of osamines. Action of carboxypeptidase released Val, Ala, Ser, and Thr from GMP and also from k-casein, therefore, the peptide must appear on the C-terminal end of the k-casein molecule. Interestingly, these workers observed that Val and Ala were liberated simultaneously and in equal amounts, suggesting that the GMP may have two chains. Carboxypeptidase liberated two new C-terminal amino acids from para-k-casein, Leu and Phe, supporting the conclusion that GMP was

released from the C-terminal end (Jollès et al., 1962).

The bond cleaved by rennin and joining the GMP to para-k-casein has been the subject of many studies. Jollès, Alais, and Jollès (1963) treated k-casein with $LiBH_4$ and obtained products similar to para-k-casein and GMP. Moreover, phenylalaninol was found in the precipitate. Consequently, an ester linkage between phenylalanine and the GMP was postulated. Garnier, Mocquot, and Brignon (1962) found that one proton was released per 55,000 g of k-casein upon the action of rennin on this protein at pH 7. Under these conditions, the release of a proton would be expected if the bond was an ester but not if a peptide linkage was involved.

Recently Beeby and Nitschmann (1963) noted a faster increase in the NANA and nitrogen soluble at pH 4.7 than that soluble in 12% TCA when low enzyme concentrations were used. They suggested that k-casein exists as a complex and that the initial action of rennin disrupts this complex freeing the NANA containing portion which is soluble at pH 4.7 but not in 12% TCA. It was proposed that a subsequent cleavage at this portion yields the GMP which is soluble in 12% TCA.

Physical Properties

Ultracentrifugal Characteristics

Polymers of approximately 13.5 S which were insensitive to temperature, ionic strength, and calcium ion concentration were formed by k-casein in buffers near neutrality (Waugh, 1958). The sedimentation coefficient did not vary more than one unit at temperatures from 1° C to 37° C and at calcium ion concentrations from 0 to 1 M (Waugh and von Hippel, 1956).

However, these authors found that by increasing the pH to 12 the polymers would dissociate, yielding a "monomer" with an S_{20} of about 1.3 S. The dissociation was reversible with respect to the sedimentation characteristics. From the ultracentrifugal and electrophoretic data on Fraction S, it was deduced that whole casein contained approximately 15% k-casein.

Similarly, Long (1958) observed sedimentation coefficients at 13.2 S to 16 S in neutral buffers and 1.1 S at pH 12. Morr (1959) reported values ranging from 12 S to 16 S for k-casein in phosphate buffer at pH 7. Coefficients of 13.9 S and 13.6 S were reported by McKenzie and Wake (1961) for their preparation in phosphate buffers at pH 6.5 and 6.9. Thompson and Pepper (1962) reported a value of 14 S for a similar preparation at pH 7.

The k-casein boundary was skewed indicating that the polymers were not entirely uniform in size or that some heterogeneity was involved (Waugh and von Hippel, 1956; Long, 1958; McKenzie and Wake, 1961). Long (1958) also observed that exposure to pH 12 caused the pattern to become more symmetrical when studied at pH 7. The molecular weight of k-casein in phosphate buffer at pH 12 was 16,300 as determined by the Archibald technique (Waugh, 1958). Using this value along with the sedimentation coefficient (1.37 S), Waugh calculated a frictional ratio for k-casein which, after making certain assumptions, indicated that the molecule could be represented by a cylinder 16 Å in diameter and 150 Å in length. However, employing the same technique and buffer system, McKenzie and Wake (1959b) obtained a molecular weight of 26,000 $\pm$ 3,000. These values should be compared to that obtained by Garnier *et al.* (1962) from proton release by rennin (55,000) and the values estimated by Beeby (1963) from a consideration of the amount and molecular weight of GMP (50,000).

Electrophoretic Characteristics

Descending mobilities obtained by free-boundary electrophoresis were -6.81 and -7.60 Tiselius units for k-casein at pH 6.98 and 6.5 in phosphate buffer, $\Gamma/2 = 0.1$, respectively (Long, 1958). Morr (1959) reported mobilities of -7.16 to -7.97 Tiselius units in phosphate buffer (pH 6.98, 0.1 $\Gamma/2$). At pH 8.4 the value was -6.8 Tiselius units.

Zone electrophoresis in urea-starch gel showed a smear directly in front of the starting slot (Wake and Baldwin, 1961: Neelin, Rose and Tessier, 1962).

PART I

THE PREPARATION AND PRELIMINARY CHARACTERIZATION OF KAPPA-CASEIN

INTRODUCTION TO PART I

When this study was initiated all the reported methods of isolation for k-casein required the use of a preparative ultracentrifuge. Since this instrument was not available at that time, a different isolation scheme was necessary. Furthermore, even when an ultracentrifuge was available, a method for obtaining crude k-casein in good yields was desirable since the capacity of the centrifuge limits the amount which can be purified.

The method of preparation developed to satisfy the above requiremetns is presented in Part I of this thesis. Also included are the characterization studies which were performed to establish the identity and homogeniety of the preparation.

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EXPERIMENTAL METHODS

Apparatus

The raw milk was collected in ten-gallon stainless steel cans. Once the volume was reduced following the precipitation of whole casein, the operations were performed in pyrex containers. Milk was separated in a small (Model 9) DeLaval disc-type separator. A refrigerated cabinet in which an ice bank was formed served as a low temperature water bath for the protein preparative operations. The pH was measured during the preparative procedures with a Beckman Zeromatic pH meter equipped with a glass electrode. A variable-speed Welch stirrer fitted with a plastic rod and blade was used to stir the protein solutions. Low speed centrifugation was performed either in an International centrifuge with a capacity of 1500 ml or a Servall type SS-1 centrifuge with a 400 ml capacity. Earlier preparations were subsequently purified by ultracentrifugation in a Beckman Model L preparative ultracentrifuge equipped with a type 40 fixed-angle rotor. In later preparations, a Model L with a high speed drive and a type 50 fixed-angle rotor was employed. Free-boundary electrophoresis was performed in a Perkin-Elmer, Model 38A Tiselius electrophoresis apparatus using circulating, refrigerated water to maintain the bath temperature at $< 2°$ C. Buffer resistances were measured with an Industrial Instruments, Model RC conductivity bridge. A Plexiglass horizontal cell equipped with a Reco power source was used for urea-starch gel electrophoresis. The analytical ultracentrifuge employed to characterize the preparations will be described in Part II of the thesis.

Chemicals and Buffers

Analytical reagent grade urea was obtained from Mallinckrodt and used without further purification. Trichloroacetic acid (TCA), reagent grade, was purchased from Fisher Scientific Company. Crystalline rennin was obtained from the Paul Lewis Laboratories and General Biochemicals; the latter's product had a reported specific activity of 960 rennin units per milligram of rennin nitrogen. Sephadex G-75 was obtained from Pharmacia. Other organic or inorganic chemicals employed were of reagent grade quality.

The buffers used in the elctrophoretic and ultracentrifugal characterization of k-casein preparations are described in Appendix I.

Preparatory Procedure

The postulation that k-casein would be more soluble in concentrated urea-TCA solutions than other caseins was prompted by the observations of Hipp *et al.* (1952) that isoelectric casein was dissociated in concentrated urea solutions and of Wake (1959) and Nitschmann and Beeby (1960) that the primary scission product of the action of rennin on k-casein was soluble in 12% TCA. Thus, if the complex dissociation was complete, some fractionation could be expected. The following experiments were designed to test this postulate.

Wet isoelectric casein (*ca* 50% dry weight) was dissolved at room temperature in concentrated urea solutions, ranging from 3 to 8 *M*, and at protein concentrations of 2 to 4%. Crystalline TCA was added in varying concentrations from 6 to 20% to aliquots of the urea solutions. The protein obtained from the supernatant following low speed centrifugation (2000 RPM for 30 min) was assayed both electrophoretically at

pH 8.6 and as a substrate for rennin. Thus, the most effective removal of non-k-casein fractions was achieved in 6.6 M urea-12% TCA solutions. Higher concentrations of urea and TCA did not appear to achieve further fractionation of the proteins. Lower concentrations of TCA resulted in less complete removal of non-k-casein fractions and lower concentrations of urea produced lower yields of crude k-casein in the supernatant.

Pooled raw milk was obtained directly from the University herd and processed immediately. The skimmilk portion was diluted with an equal volume of distilled water and isoelectric casein obtained by adjusting the pH to 4.6 through the slow additions of 1 N HCl. The precipitated casein was redispersed in water in its original concentration and dissolved by the slow addition of 1N NaOH, being careful not to exceed pH 8. Again, the dissolved casein was isoelectrically precipitated and washed with several liters of 1 : 1 (v/v) mixture of ethyl ether and ethanol. Finally, the washed casein was redissolved and reprecipitated to assure complete elimination of serum proteins. Calcium-insensitive casein fractions were prepared from the whole casein using four variations of the urea-TCA method. Careful stirring was observed in all of the preparations to avoid foaming and surface denaturation. The final product was lyophilized and stored at -20° C.

Isolation Procedure for Preparation No. 1

Isoelectric casein was dissolved in 6.6 M urea at a concentration of 2 - 4% and the temperature lowered to 1 - 3° C in the ice bath. Crystalline TCA was added slowly with stirring to a final concentration of 12% by weight. After standing for 30 min, the precipitated casein fraction was removed by centrifugation in the cold room (5 - 10° C at

1000 X G for 30 min). The pH of the supernatant was adjusted to 7 at 3° C through the slow addition of solid NaOH. The neutralized solution was dialyzed to remove the resulting neutralization salts. The solution was concentrated to 1 - 2% protein by pervaporation, cooled to 3° C and adjusted to pH 8. Then, 2 M $CaCl_2$ was added dropwise to give a final concentration of 0.25 M. After allowing the mixture to stand overnight, much of the calcium-sensitive precipitate had settled out. The remaining precipitate was removed by centrifugation in the cold room as previously described. Upon warming the supernatant to 30° C, more precipitate formed and was removed by centrifugation at 25,000 X G for 10 min in the Servall. Calcium chloride was removed from the clear supernatant by dialysis. The protein concentration of the dialyzed supernatant was again adjusted to 1 - 2% by pervaporation. At 3° C, the pH was adjusted to 11.3 momentarily to dissociate the complexes and then rapidly lowered to 4.4 with cold 0.2 N HCl to precipitate the k-casein-rich fraction. The precipitate was collected by centrifugation (1000 X G for 10 min), dissolved with 0.1 N NaOH (pH < 8), dialyzed, and lyophilized. A schematic of the preparatory procedure is presented in Figure 1.

Isolation Procedure for Preparation No. 2

The protein was prepared in the same manner as Preparation No. 1 except that the operations were performed at room temperature. Also, Step 6 of the isolation scheme in Figure 1, consisting of the pH adjustment and isoelectric precipitation, was omitted.

Isolation Procedure for Preparation No. 3 and 3 A

Approximately equal amounts of isoelectric casein and crystalline

urea were mixed and made to a final volume in which the urea concentration was 6.6 M. The pH (ie. about 5) was adjusted to 4.7 with 1 N HCl. When the casein was completely dissolved, the solution was diluted to a 3.3 M urea concentration by dropwise addition of distilled water. There was no visible precipitate at 4.63 M urea concentration as reported by Hipp et al. (1952). A similar observation was made by Nielsen (1957). After standing for 1 - 2 hours, a viscous, sticky precipitate (α-casein fraction) had settled out and most of the supernatant was decanted. The remaining mixture was centrifuged at 1000 X G for 20 - 30 min. The precipitate was redissolved in 6.6 M urea (pH 4.7) and the final volume of the solution determined. Sufficient water was added dropwise, as before, to give a 4.0 M urea concentration. The precipitate was collected by centrifugation (1000 X G for 20 min) and the step repeated. The final precipitate was redissolved in sufficient 6.6 M urea at 3° C to give a protein concentration of 2 - 4%. From this point, the procedure described for Preparation No. 1 was followed. The complete procedure is shown schematically in Figure 1.

Additional Purification as Applied to Preparations No. 3 and 3 A

Portions of the preparations made by the procedure for 3 and 3 A were further purified according to the following scheme. Crude k-casein solutions (2 - 3% protein) were made 0.1 M with respect to NaCl at pH 7 and centrifuged for 5 - 6 hours at 40,000 or 50,000 RPM and 0 - 4° C in a Spinco Model L. The bottom 1/3 to 1/4 of the liquid column (a bottom layer was clearly visible), including a small amount of pellet, represented purified k-casein. For most of the physical studies reported in Part II of this thesis, k-casein (Preparation No. 3 A) was further

purified by elution from a Sephadex G-75 column equilibrated with 0.1 M NaCl. The column was monitored at 280 mμ. k-Casein was eluted in the eluate immediately following the void volume.

Isolation Procedure for Preparation No. 4

In earlier preparations, some difficulty was experienced in removing β-casein; therefore, the purification procedure for the α-casein fraction described by Hipp et al. (1952) was used. The crude α-casein fraction, initially obtained by diluting solutions in 6.6 M urea to 3.3 M urea concentration, was dissolved in 6.6 M urea solutions containing 10.6 g/l NaCl. The α-casein fraction was precipitated by dilution to 3.3 M urea concentration and the step repeated. In other respects, the preparatory procedure was the same as for Preparation No. 3 and 3 A.

However, the final product (from Step 6 of Figure 1) was obtained in yields of only 0.1 - 0.2% of the whole casein. Furthermore, characterization studies indicated that this fraction did not contain k-casein.

Analytical Methods

Electrophoretic mobilities were calculated from measurements made from the position of the initial boundary on the descending and ascending patterns, as follows:

$$\mu = \frac{d\ A\ K}{tIRm}$$

where μ is the electrophoretic mobility in $cm^2\ volt^{-1}\ sec^{-1}$, d the distance the boundary traveled in cm, A the cross-sectional area of the electrophoresis cell in cm^2, K the conductivity cell constant, t the time in seconds, I the current in amperes, R the resistance of the buffer in

ohms, and m the magnification of the optical system. The buffer resistance was determined by placing the conductivity cell containing the buffer in the electrophoresis water bath just prior to or following the experiment. Relative amounts of the various components were determined from the relative refractive areas measured, with the aid of a planimeter, from tracings of the patterns enlarged five times. Boundaries were divided by drawing a vertical line from the minimum gradient between the peaks to the base line. The isoelectric point of k-casein was estimated from a plot of mobility (μ) against the pH.

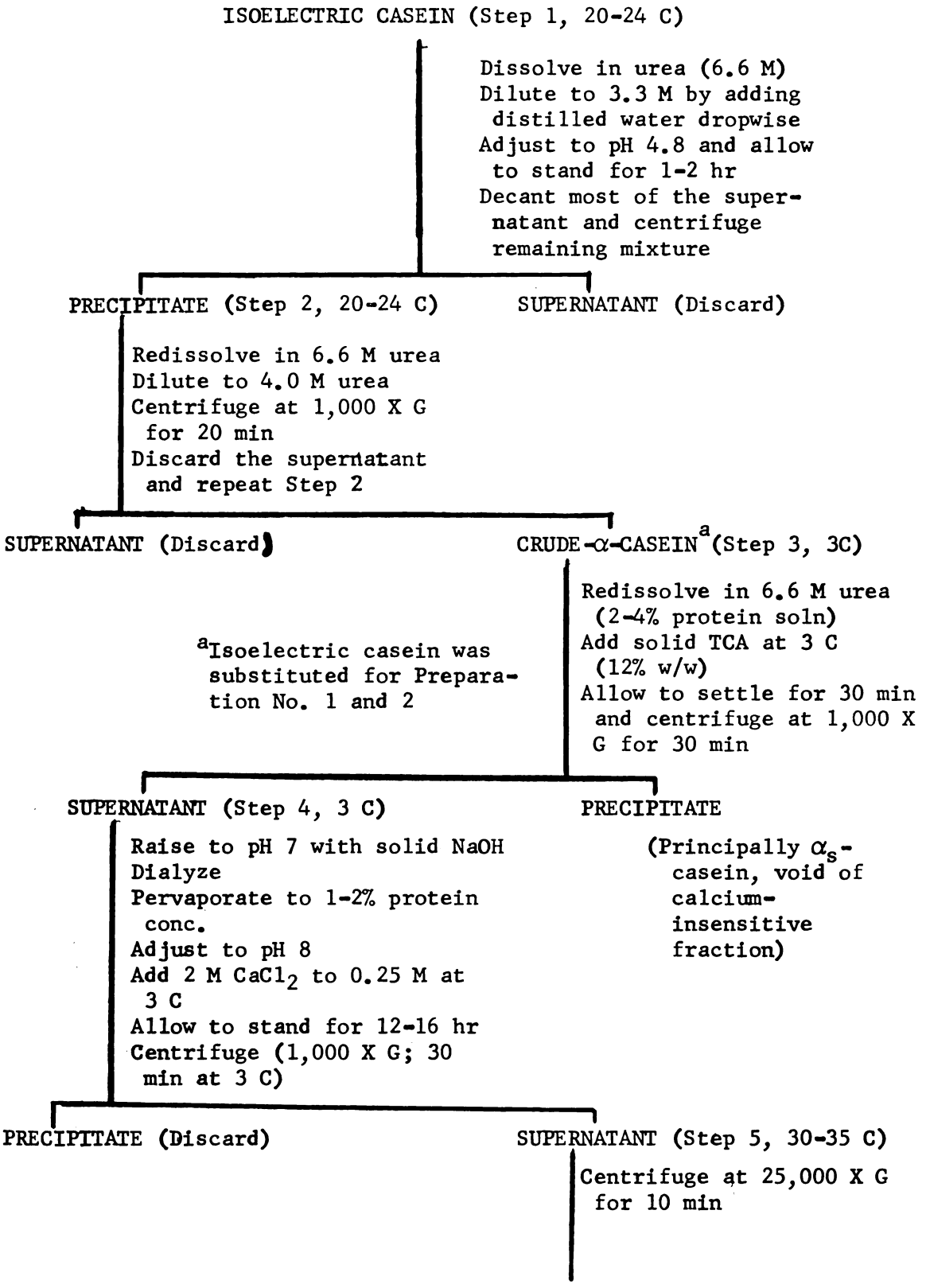
An estimate of the isoelectric point was also made by the minimum solubility method. A series of acetate buffers were prepared ranging in pH from 3.6 to 4.5. Five milliliters of a 2% k-casein solution were added to 5 ml of buffer, possessing a concentration of salts such that the final solution had the composition: 0.02 M sodium acetate and 0.08 M NaCl. The pH of the buffer was determined before and after addition of the protein. The solution was equilibrated at 5° C with intermittant mixing for 48 hours. The amount of protein remaining in the supernatant following centrifugation at 3000 X G for 10 min at 5° C was determined a) spectrophotometrically at 280 mμ following the addition of base to give a clear solution and b) by the conventional macro-Kjeldahl procedure.

The ability of k-casein to stabilize α_s-casein in the presence of calcium ion was evaluated by the method of Zittle (1960). The final test solution consisted of 10 ml of 0.02 M $CaCl_2$, 10 mg/ml of α_s-casein, and varying amounts of k-casein. The solution was prepared as follows: 5 ml of 2% α_s-casein was mixed with varying amounts of a 1% solution of k-casein; then 2 ml of 0.1 M $CaCl_2$ was added and the solution was made

to 10 ml. After holding for 30 min at 30° C, the mixture was centrifuged at 3000 X G for 5 min. The 280 mμ absorption of 1 ml of supernatant diluted to 25 ml with 1 ml of 0.1 N NaOH and redistilled water was determined. The concentration of protein remaining in the supernatant was obtained from a standard curve prepared by measuring known concentrations of α_s-casein. Since all of the k-casein was in the supernatant, the amount of α_s-casein present in the stabilized complex was obtained by subtracting the per cent k-casein in the test solution from the total protein.

Zonal electrophoretic analyses were performed in urea-starch gels according to the method described in detail by Wake and Baldwin (1961). A discontinuous buffer system was used and consisted of Tris-citrate buffer incorporated into the gel and borate buffer in the buffer compartments. The gel was made to 7 M with respect to urea. Electrophoresis was carried out in a cold room ($\sim$ 5° C). The resolved proteins were stained with Amido Black.

Ultracentrifugal characterization and determination of purity were performed at temperatures ranging from 3 to 25° C in pH 7 phosphate buffer ($\Gamma/2 \cong 0.1$ or 0.2) using a rotor speed of 59,780 RPM. Both a regular analytical and a synthetic boundary cell were used. The position of the maximum ordinate was used to calculate the sedimentation coefficient (see Appendix II).



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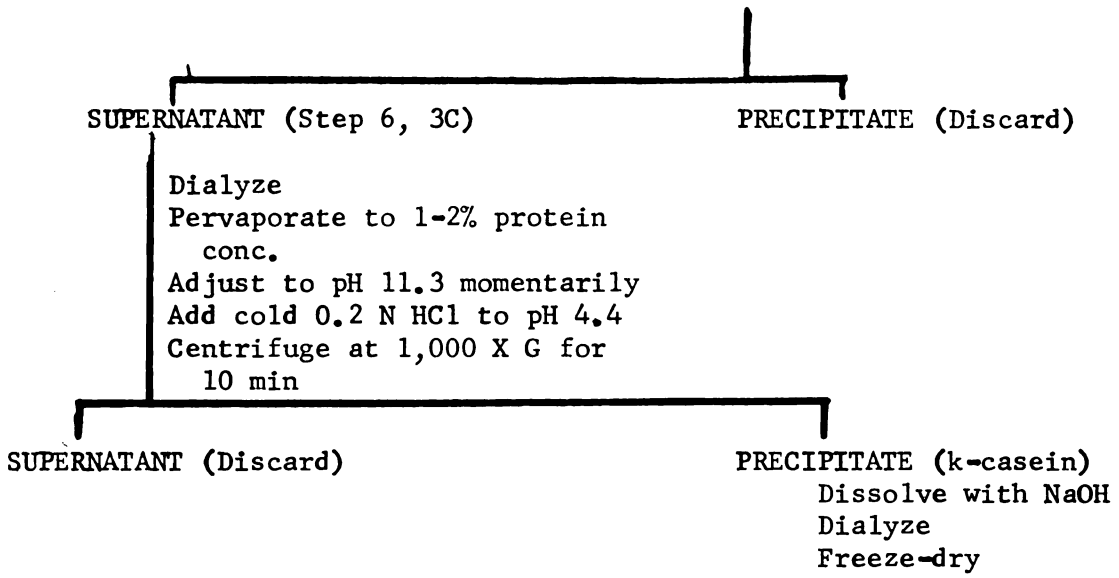

Additional purification a) centrifuge crude k-casein dissolved in 0.1 M NaCl at pH 7 for 6 hours at 50,000 RPM and 4° C.
b) collect the bottom layer and pellet from a) and elute this portion from a Sephadex G-75 column equilibrated with 0.1 M NaCl.

Figure 1. Procedure followed for obtaining k-casein from a concentrated urea-TCA solution of isoelectric casein or crude α-casein fraction.

RESULTS

Preparation and Yields

The complete scheme for the preparation of k-casein is shown schematically in Figure 1. α-Casein fraction was obtained in yields of nearly 50% of the starting whole casein. As shown in Figure 2 (A and B), α-casein No. 3 contained some β-casein, but α-casein No. 3 A appeared electrophoretically homogeneous. Precipitation in 6.6 M urea with 12% TCA removed from solution approximately 40% of the initially dissolved α-casein fraction or whole casein. With whole casein, large amounts of α_s-, β-, and possibly γ-casein were precipitated (Figure 2 E). The precipitate obtained from Hipp's α-casein fraction was essentially α_s-casein. The electrophoretic and sedimentation-velocity patterns in Figure 2 (C and D) indicate that some β-casein was present. Subsequent preparations obtained from β-casein free α-casein fraction were homogeneous as measured by free-boundary electrophoresis. Further, this precipitated protein was established as α_s-casein by its sedimentation coefficient (S_{20} = 4.9 S in phosphate buffer, pH 7, 0.1 $\sqrt{\mu}$/2), electrophoretic mobility (μ = -7.2 Tiselius units in veronal buffer, pH 8.6, 0.1 $\sqrt{\mu}$/2), instability to calcium ion (0.02 M $CaCl_2$ caused a nearly complete precipitation), and the fact that it formed micelles and was stabilized with k-casein in the presence of calcium ion.

Approximately 70% of the material which was soluble in urea-TCA was removed by addition of calcium ion. The properties of this fraction were identical to those of the urea-TCA insoluble material. Thus α_s-casein appeared to be divided between the urea-TCA precipitate and the calcium ion precipitate, with slightly more precipitating in the urea-

TCA. Due to the selective precipitation of α_s-casein in urea-TCA, the urea-TCA soluble portion was greatly enriched with respect to k-casein. However, as shown by the electrophoretic patterns in Figure 2 (G and H), a complex of α_s- and k-casein still existed.

Electrophoretic, ultracentrifugal, and yield characteristics of k-casein preparations No. 1, 2, and 3 are recorded in Table 1 and Figure 3. These data illustrate the differences in composition and properties between the preparations obtained directly from whole casein (No. 1 and 2) and that fractionated from crude α-casein fraction (No. 3). Electrophoretic resolution in neutral or alkaline buffers indicated that preparation No. 3 contained the least amount of discernible β-casein. At pH 2.3, the preparations appeared polydispersed, probably due to protein-protein interactions. Sedimentation under similar conditions revealed the same polydispersity. The room temperature preparation from whole casein was obtained in the highest yield. However, it contained only 60% of a fast sedimenting component at pH 7, whose S_{20} was higher than that previously reported for k-casein (20.6 S as opposed to 13.5 S). Preparation No. 1 (prepared at 3 - 5° C) closely resembled Fraction S, reported by Waugh and von Hippel (1956). About 90% of the refractive area in electrophoretic patterns and 77% of the area in sedimentation-velocity patterns corresponded to k-casein, indicating the presence of β- and possibly λ-caseins.

When α-casein fraction was employed as the starting material, crude k-casein (90 - 92% purity) was obtained from Step 6 of the fractionation scheme shown in Figure 1. Yield approximating 30 - 50% of the theoretical yield, assuming k-casein to be 15% of the whole casein, were obtained. Sedimentation-velocity patterns of this fraction in phosphate buffer

(pH 7) are shown in Figure 4, Row A, and the calculated sedimentation coefficients are given in Table 2. Sedimentation patterns of Preparations No. 3 and 3 A which were purified by preparative ultracentrifugation are represented in Row B. The sedimentation coefficient of the sedimented pellet was slightly higher than that of other k-casein preparations even though the purity was not greater. Ultracentrifugal patterns of Preparation No. 3 A, purified by both ultracentrifugation and elution from G-75 Sephadex, are shown in Row C. For comparison, patterns of a preparation by the method of Waugh and von Hippel (1956), purified by ultracentrifugation and elution from G-75 Sephadex, together with a preparation by the method of McKenzie and Wake (1961)[1] are also shown. The first two patterns demonstrate the superiority of the synthetic boundary cell over the conventional analytical cell as a means of detecting small quantities of λ-casein in the k-casein preparations. The third and fourth patterns in Row C represent purified Fraction S and the McKenzie-Wake preparation, respectively. The data from these experiments, listed in Table 2, indicated that the k-caseins obtained by the three different methods were essentially the same. Urea-starch gel electrophoresis of purified No. 3 A and the McKenzie-Wake preparation also showed similar results (Figure 5, Column 4). As shown in Figure 6, the free-boundary electrophoretic patterns of purified No. 3, No. 3 A and purified No. 3 A were indicative of homogeneity in neutral phosphate buffer.

1 This preparation was provided by Dr. M. P. Thompson of Eastern Regional Research Laboratory (USDA).

Free-Boundary Electrophoretic Characteristics

Electrophoretic analyses of Preparation No. 3 were performed over a pH range of 2.3 to 8.6. The results are recorded in Table 3 and selected typical patterns are shown in Figure 7. A split peak similar to that previously described for k-casein at pH 2.3 was observed at pH 5.5 and 6.0 but to a lesser extent and restricted to the ascending patterns. Also, the area of the peak ascribed to β-casein was smaller at pH 5.5 and 6.0 (4 and 5% respectively) than that at pH 6.5 (7.5%). The area under the slowly migrating peak decreased as the pH was increased from 6.5 to 8.6 (with the exception of pH 8.3).

The descending mobilities (μ) for the principle component recorded in Table 3 were plotted as a function of buffer pH, Figure 8. The solid line connects the experimental values and intercepts the point of zero mobility at ca pH 3.8. The broken line passes through the three points nearest to the isoelectric point and intercepts the axis at pH 4.1.

pH of Minimum Solubility at 0.1 Ionic Strength

The isoelectric point of Preparation No. 3 was determined from the pH of minimum solubility. Absorption at 280 mμ and nitrogen in mg/ml for the k-casein which remained in the supernatant are shown as a function of pH in Figure 9. These data indicated that at 4-5°C a minimum amount of the protein remained soluble between pH 3.8 and 4.1.

Stabilization of α_s-Casein

The ability of Preparation No. 3 to stabilize α_s-casein in the presence of calcium ion at 30° C is shown in Figure 10. At a k-/α_s-casein

ratio of 1 : 10, approximately 90% of the α_s-casein was stabilized.

Complex Formation with α_s-Casein

Electrophoretic and sedimentation characteristics of α-casein fraction prepared by the method of Hipp et al. (1952) were compared with those for two k-/α_s-casein complexes formed by two different treatments of a 1 : 4 (w/w) mixture of k- and α_s-casein. In the first method, the mixture containing 1% protein was adjusted to pH 12 for 30 min at room temperature, followed by neutralization and dialysis against pH 7.5 phosphate buffer prior to analysis. The second procedure consisted of dissolving the mixture in 6.6 M urea solution, adjusting the pH to 4.8, and then diluting the solution to 3 M with respect to urea. The precipitate obtained was dialyzed against water to remove the urea and then against the phosphate buffer (pH 7.5).

The patterns in Figure 11 show that qualitatively the complexes were similar to the α-casein fraction. α-Casein fraction and the complex formed by pH adjustment had the same electrophoretic mobility (-7.6 to -7.7 Tiselius units), whereas that formed in urea was greater (-9.0 Tiselius units). The sedimentation coefficients were 4.3 S, 6.2 S, and 4.7 S, respectively, for the α-casein fraction, the complex formed by pH adjustment, and the complex formed in urea.

Characteristics of the λ-Casein Fraction (Preparation No. 4)

When the α-casein fraction was purified by precipitation from urea solutions containing NaCl as described in the procedure for obtaining Preparation No. 4, only small quantities of final product were obtained (Step 6 of Figure 1). Further, the protein was not characteristic of

k-casein when subjected to urea-starch gel electrophoretic analysis (Figure 5). The fraction was not sensitive to calcium ion, contained 1.2% phosphorus and was not a primary substrate for rennin as demonstrated by qualitative observations and ultracentrifugal and electrophoretic properties. Also, the fraction was incapable of stabilizing α_s-casein to calcium ion. The amino acid composition is compared to that for k-casein in Appendix III.

Although the fraction was heterogeneous as shown by urea-starch gel electrophoresis, free boundary electrophoresis and sedimentation-velocity in pH 7 phosphate buffer revealed only one symmetrical boundary. The mobility measured from the descending pattern was -6.86 Tiselius units and the sedimentation coefficient for a 6 mg/ml solution was 1.68 S. Moreover, molecular weights in phosphate buffer (pH 7, 0.1 Γ/2) obtained by the Archibald method (Schachman 1957) did not indicate weight heterogeneity ($M_w \approx 40,000$). However, short column equilibrium analysis (see Appendix II) in 5 M guanidine · HCl was indicative of heterogeneity, giving M_w = 20,000 and M_z = 90,000.

Action of Rennin on k-Casein Preparations

Studies of the primary rennin action were made on three preparations, two of which were purified and one of which was similar to Waugh's Fraction S (ca 70% k-casein). When a purified sample of k-casein (Preparation No. 3 A) was reacted with rennin (1 - 3 μg/ml) in redistilled water, the solution first appeared cloudy (1 - 5 min), then a precipitate formed which settled out upon standing. Under the same experimental conditions, the crude k-casein did not become cloudy. The addition of NaCl to 0.1 M caused the solution to become opalescent. Still a precipitate did not form. A peptide was released as shown by

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the increase in non-protein nitrogen (NPN = 12% TCA soluble N) observed during the reaction, ie. 4.3%. The increase in NPN (6.7%) reported by Wake (1959) for pure k-casein gave a calculated purity for this preparation of 65%, which was in agreement with the ultracentrifugal data.

The soluble peptides and para-k-casein were obtained from both purified Preparations No. 3 and No. 3 A by centrifuging the neutral (ca pH 7) mixture in a Spinco Model L at 40,000 RPM for 3 hours at 4° C in one instance and at 50,000 RPM for 2 hours at room temperature in the other. The rennin action was stopped by heating to 80° C for 10 sec. in the case of Preparation No. 3 and by adjusting the pH to 9.5 with ammonium hydroxide and allowing the mixture to stand for 2 hours at room temperature in the case of Preparation No. 3 A. Following centrifugation the supernatant and pellet were quantitatively recovered and lyophilized without dialysis (both reactions were performed in redistilled water) to avoid the loss of possible small peptides.

Preparation No. 3 (1.46 g) yielded 333 mg of soluble peptides and 814 mg of para-k-casein. Starting with 50 mg of 3 A, 15 mg of soluble peptides and 34 mg of para-k-casein were obtained. Thus about 30%, by weight, of the k-casein appeared, as soluble peptides in both cases. This value gave approximately 23% of the total nitrogen as soluble nitrogen assuming 15.3% N for k-casein and 11.7% N for the peptides (Brunner and Thompson, 1959).

The weight average and Z-average molecular weights of the soluble peptides from purified 3 A were determined by the short-column equilibrium method (see Appendix II) in veronal buffer (pH 8.6, 0.1 Γ/2) at 25.0° C using a rotor speed of 29,500 RPM. For a concentration of 4.8 mg/ml, M_w was calculated to be 5900 and M_z, 6000, using 0.68 for the

partial specific volume (Brunner and Thompson, 1959). The method of Sophianopoulos et al. (1962) (see Appendix II) was used to determine the diffusion coefficient. A value of 10.0 Ficks was obtained.

Preliminary observations showed that rennin was capable of coagulating skimmilk in the presence of diisopropylflourophosphate. However, in the presence of 1% sodium dodecyl sulfate (SDS), k-casein was not effected by rennin as shown by NPN liberation experiments.

TABLE 1

Comparison of the electrophoretic, ultracentrifugal, and yield characteristics of k-casein preparations

Identification of k-casein preparation	Properties of the electrophoretic peaks[a]						Sedimentation-velocity[c] values for leading boundary		
	Buffer[b]								
	Veronal pH 8.6		Phosphate pH 7.0		HCl:NaCl pH 2.4		S_{20}	Concentration (A%)	Yield[d] (%)
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2			
Preparation No. 1 TCA added to solution of urea and isoelectric casein at 2-4 C	μ[e] = -7.4	-4.6	-6.6	-3.7	+3.4	+1.4	12.7	77	
	A%[f] = 90	10	96	4	83	17			8.9
Preparation No. 2 TCA added to solution of urea and isoelectric casein at 21-24 C	μ = -6.2	-3.6	-6.2	-2.6	+2.0	+1.2		58	
	A% = 90	10	89	11	85	15	20.6		15
Preparation No. 3 TCA added to solution of urea and crude α-casein at 2-4C	μ = -6.7	-3.4	-7.5	-3.2	+2.1	+1.2	12.9	92	
	A% = 99	1	97	5	76	24			8.3

Preparation No. 3									
TCA added to solution of urea and	II	= -6.7	-3.4	-7.5	-3.2	+2.1	+1.2	12.9	92
	A2	= 93	1	67	5	26	24		8.3

TABLE 1 - Continued

[a] Fast component was presumed to be the k-casein fraction.

[b] Concentration of protein was 1.0%.

[c] Protein was carried in phosphate buffer at pH 7.0, $\Gamma/2 = 0.1$, at 3 C.

[d] Expressed as the percentage of total isoelectric casein.

[e] Mobilities were measured from descending pattern.

[f] Relative areas were measured from descending pattern.

TABLE 2

Quantitative characteristics of the sedimentation-velocity patterns of the k-caseins shown in Figure 4a

Preparation	Concentration (mg/ml)	$\Gamma_{/2}$	S_{20} X 10^{13}	% of total refractive area
No. 3	6	0.1	12.9	92
No. 3A	6	0.1	--	90
Prepared by E. M. McCabe	10	0.2	13.2	90
No. 3 purified by ultracentrifugation	6	0.1	14.5	96
No. 3A, bottom layer obtained from ultracentrifugation	6	0.2	13.1	95
No. 3A, pellet obtained from ultracentrifugation	5	0.2	16.6	93
No. 3A, purified by ultra-centrifugation and elution from G-75 Sephadex	8.2	0.2	13.4	97
k-Casein prepared by the McKenzie-Wake method	10	0.1	13.4	97
Fraction S purified by ultracentrifugation and elution from G-75 Sephadex	6	0.2	15.0	--

[a] Experiments were performed in pH 7.0 phosphate buffer.

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TABLE 3

Electrophoretic properties of k-casein (Preparation No. 3) in buffers from pH 2.3 to 8.6

Buffer System ($\Gamma/2 = 0.1$)	pH	Protein concentration (%)	Electrophoretic characteristics: Mobility $\mu X10^5$ $cm^2 sec^{-1} v^{-1}$ Descending	Mobility $\mu X10^5$ $cm^2 sec^{-1} v^{-1}$ Ascending	Refractive area[a] (%) Fast peak	Refractive area[a] (%) Slow peak
HCl:NaCl	2.3[b]	2.0	+2.4[c]	+3.2[c]	100°	0
			+0.8[d]	+2.2[d]		
Acetate	4.5[b]	0.6	-3.0	-3.1	...	...
Acetate	4.6	0.6	-4.4	-4.4	92.7	7.3
Acetate	4.7	0.6	-4.6	-4.7	...	...
Acetate	5.1	0.6	-6.3	-6.5	...	...
Acetate	5.3	0.6	-6.2	-6.6	...	...
Acetate	5.5[b]	0.6	-6.4	-6.4[d]	96.2	3.8
				-5.9[e]		
Phosphate	6.0[b]	0.6	-6.8	-7.6[d]	95.1	4.9
				-7.4		
Phosphate	6.5	0.6	-7.2	-7.4	92.5	7.5
Phosphate	6.7	0.6	-7.7	-8.1	93.7	6.3
Phosphate	7.0[b]	0.6	-8.2	-8.5	94.7	5.3
Phosphate	7.1	0.6	-7.6	-8.1	97.7	2.3
Phosphate	7.5	0.6	-8.6	-8.9	...	...
Veronal:HCl	8.3	0.6	-7.5	-7.6	95.0	5.0
Veronal	8.6[b]	1.0	-6.7	-6.8	99.0	1.0

[a]Measured from the descending patterns.
[b]Electrophoretic patterns for these analyses are shown in Figure 7. Higher concentrations of protein are required at pH 2.3 to give comparable refractive areas.
[c]Mobility of the leading portion of the divided peak.
[d]Mobility of the trailing portion of the divided peak.
[e]Combined area of divided peak.

Figure 2. Electrophoretic and sedimentation patterns of the material obtained from various steps of the isolation procedure. Only the ascending electrophoretic boundaries are shown. The studies were performed in veronal buffer (pH 8.6, 0.1 $\Gamma/2$) except for D and G which were in phosphate (pH 7, 0.1 $\Gamma/2$). A- α-casein fraction, Preparation No. 3. B- α- casein fraction, Preparation No. 3 A. C- TCA precipitate, Preparation No. 3. D- TCA precipitate, Preparation No. 3; sedimentation velocity, 59,780 RPM, 20° C. E- TCA precipitate, Preparation No. 1. F- Calcium ion precipitate, Preparation No. 3. G- TCA soluble fraction, Preparation No. 1. H- TCA soluble fraction, Preparation No. 3.

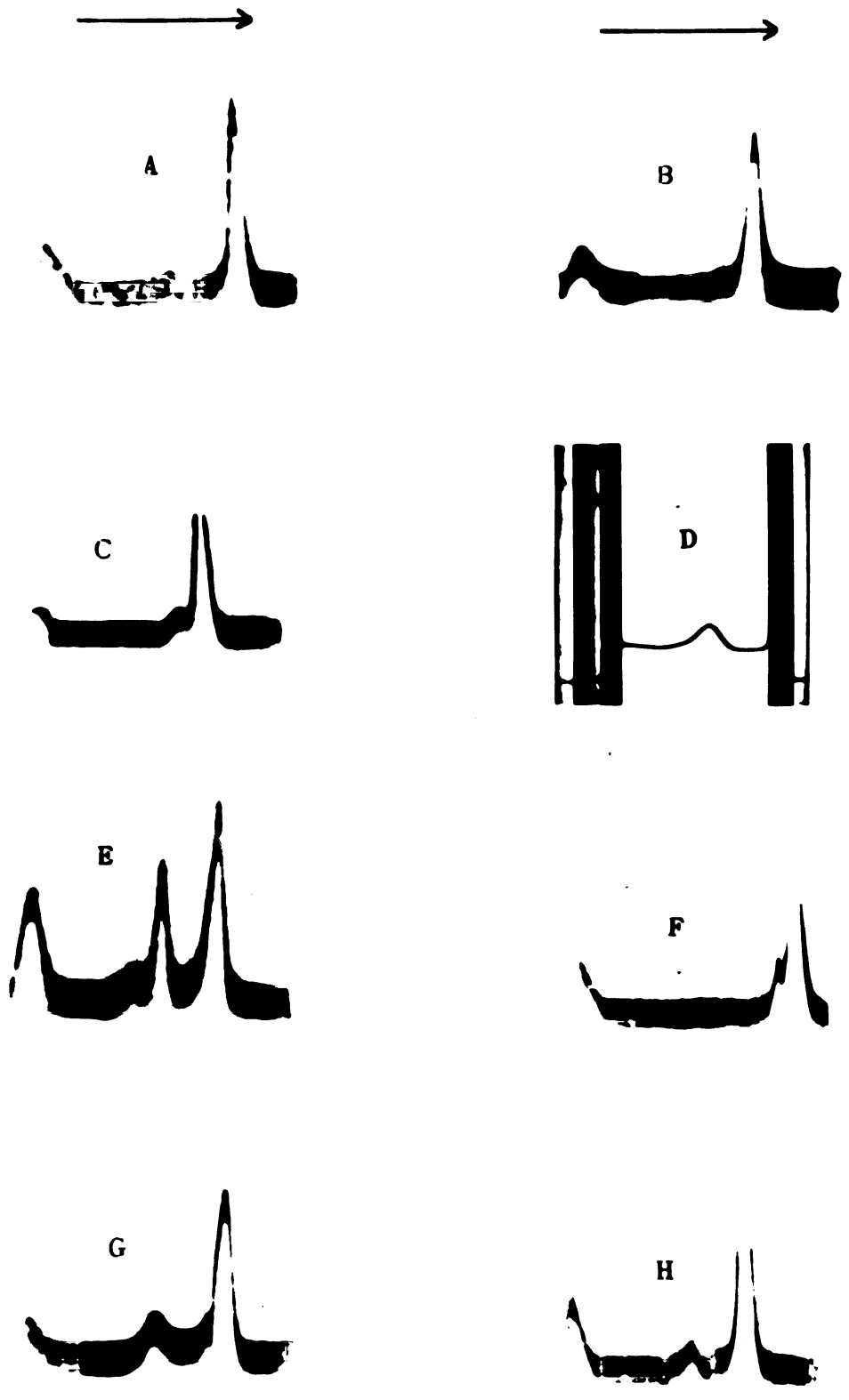

Figure 2

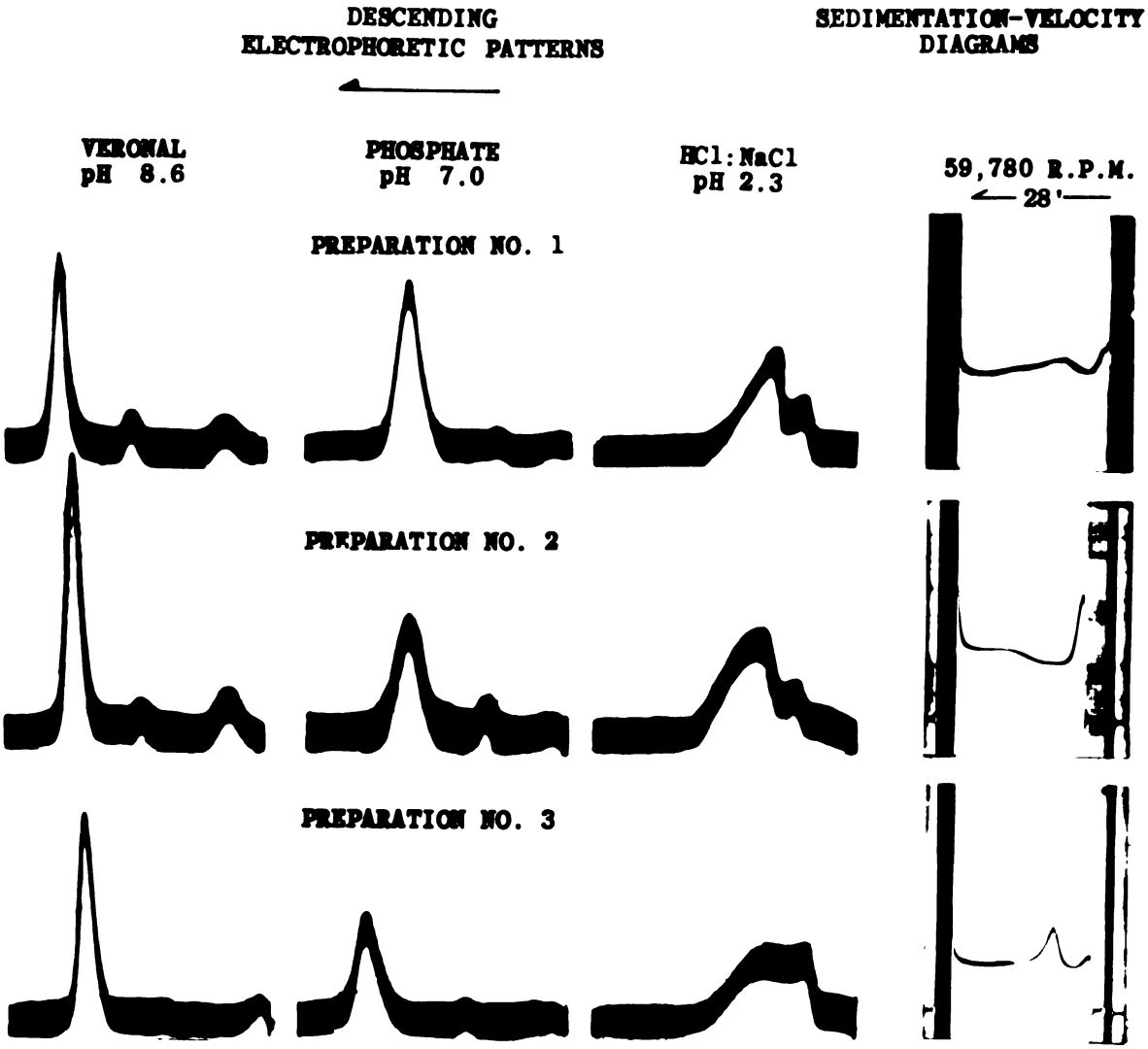

Figure 3. Electrophoretic and sedimentation-velocity patterns of the k-casein preparations reported in Table 1. Ultracentrifugation was conducted in phosphate buffer (pH 7.0) at 3° C.

Figure 4. Sedimentation-velocity patterns of different k-casein preparations in pH 7.0 phosphate buffer. The rotor speed was 59,780 RPM. The first patterns in rows A and B and the middle two patterns in row C were obtained from a regular analytical cell. The other patterns were obtained from experiments using the synthetic boundary cell. Row A (left to right) - Preparation No. 3, 0.1 $\sqrt{}/2$ buffer, 2° C, 36 min.; Preparation No. 3 A, 0.1 $\sqrt{}/2$ buffer, 5.1° C, 28 min.; Prepared by E. M. McCabe according to the procedure given in Figure 1, 0.2 $\sqrt{}/2$ buffer, 25° C, 16 min. Row B (left to right) - Ultracentrifugally purified preparation No. 3, 0.1 $\sqrt{}/2$ buffer, 3° C, 36 min.; Preparation No. 3A, bottom layer from preparative ultracentrifugation, 0.2 $\sqrt{}/2$ buffer, 20° C, 16 min.; Preparation No. 3 A, pellet from preparative ultracentrifugation, 0.2 $\sqrt{}/2$ buffer, 20° C, 16 min. Row C (left to right) - Preparation No. 3 A, purified by ultracentrifugation and elution from Sephadex G-75, 0.2 $\sqrt{}/2$ buffer, 25° C, 20 min.; same as previous protein, 0.2 $\sqrt{}/2$ buffer, 25° C, 24 min.; Fraction S purified as above, 0.2 $\sqrt{}/2$ buffer, 25° C, 16 min.; k-casein prepared by the McKenzie-Wake method, 0.1 $\sqrt{}/2$ buffer, 24° C, 16 min.

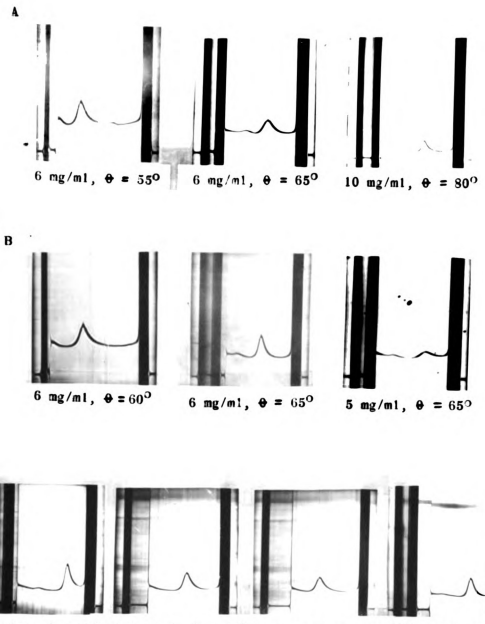
A
6 mg/ml, θ = 55°
6 mg/ml, θ = 65°
10 mg/ml, θ = 80°
B
6 mg/ml, θ = 60°
6 mg/ml, θ = 65°
5 mg/ml, θ = 65°
C
10 mg/ml, θ = 70°
3.2mg/ml, θ = 70°
6.2 mg/ml, θ = 70°
10 mg/ml, θ = 70°

Figure 4

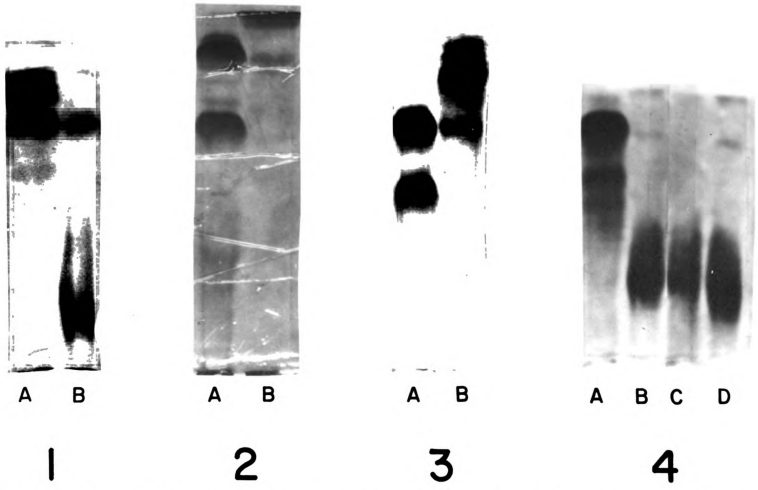

Figure 5. Urea-starch gel electrophoretic patterns of α_s-, k-, and λ-caseins. Column 1: A) λ-casein fraction, B) k-casein, Preparation No. 3. Column 2: A) whole casein, B) λ-casein fraction, Column 3: A) calcium-sensitive casein, B) λ-casein fraction. Column 4: A) TCA-precipitate, Preparation No. 3, B) k-casein, purified Preparation No. 3A, C) k-casein prepared by the McKenzie-Wake method, D) k-casein, purified Preparation No. 3A. Note: The pattern in Column 3 and the McKenzie-Wake k-casein were a gift from Dr. M. P. Thompson of Eastern Regional Research Laboratory (USDA).

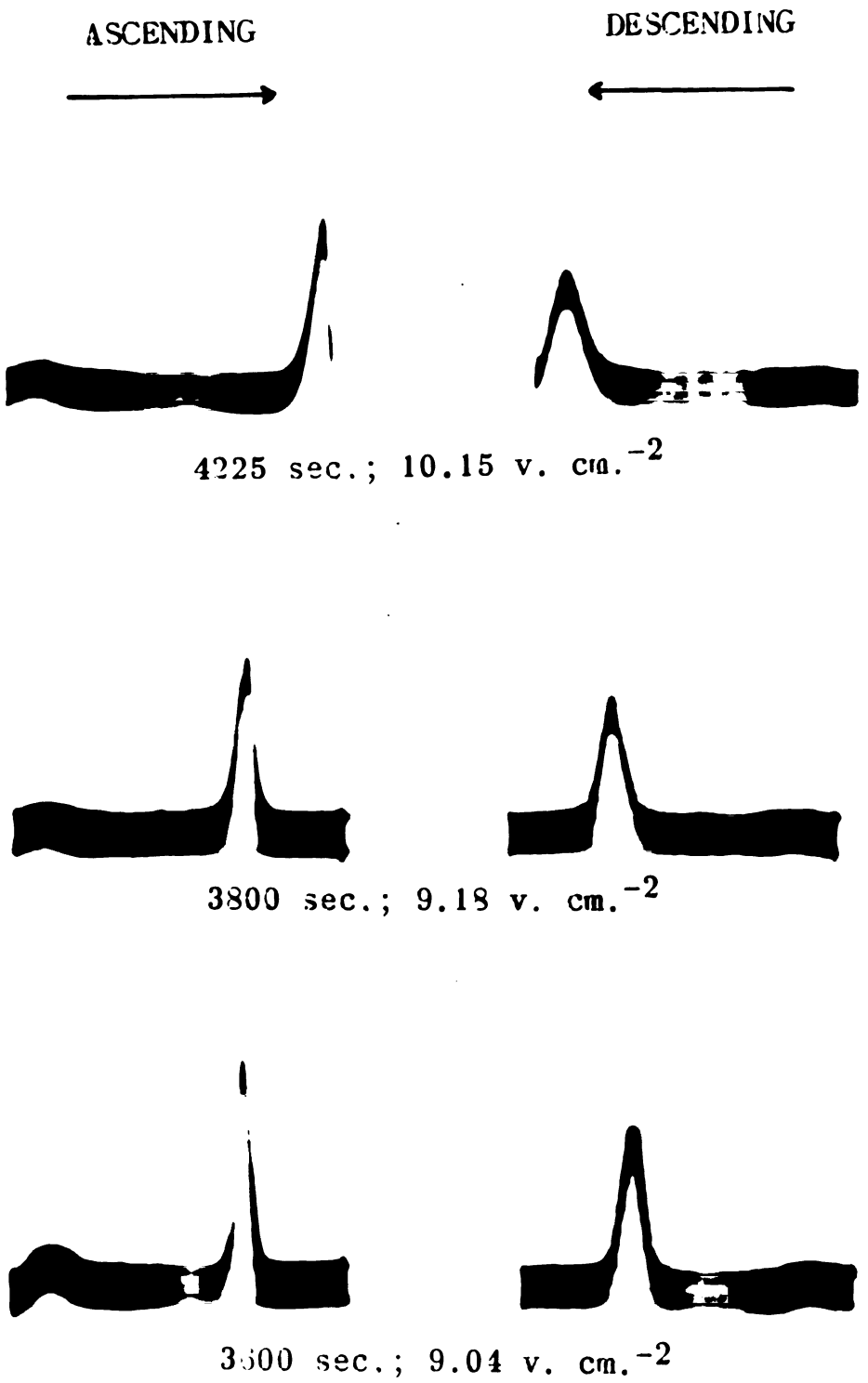

Figure 6. Free-boundary electrophoretic patterns of purified k-caseins in pH 7.0 phosphate buffer, $\Gamma/2 = 0.1$. Row A - Preparation No. 3, purified by ultracentrifugation. Row B - Preparation No. 3A. Row C - Preparation No. 3A, purified by ultracentrifugation and elution from Sephadex G-75.

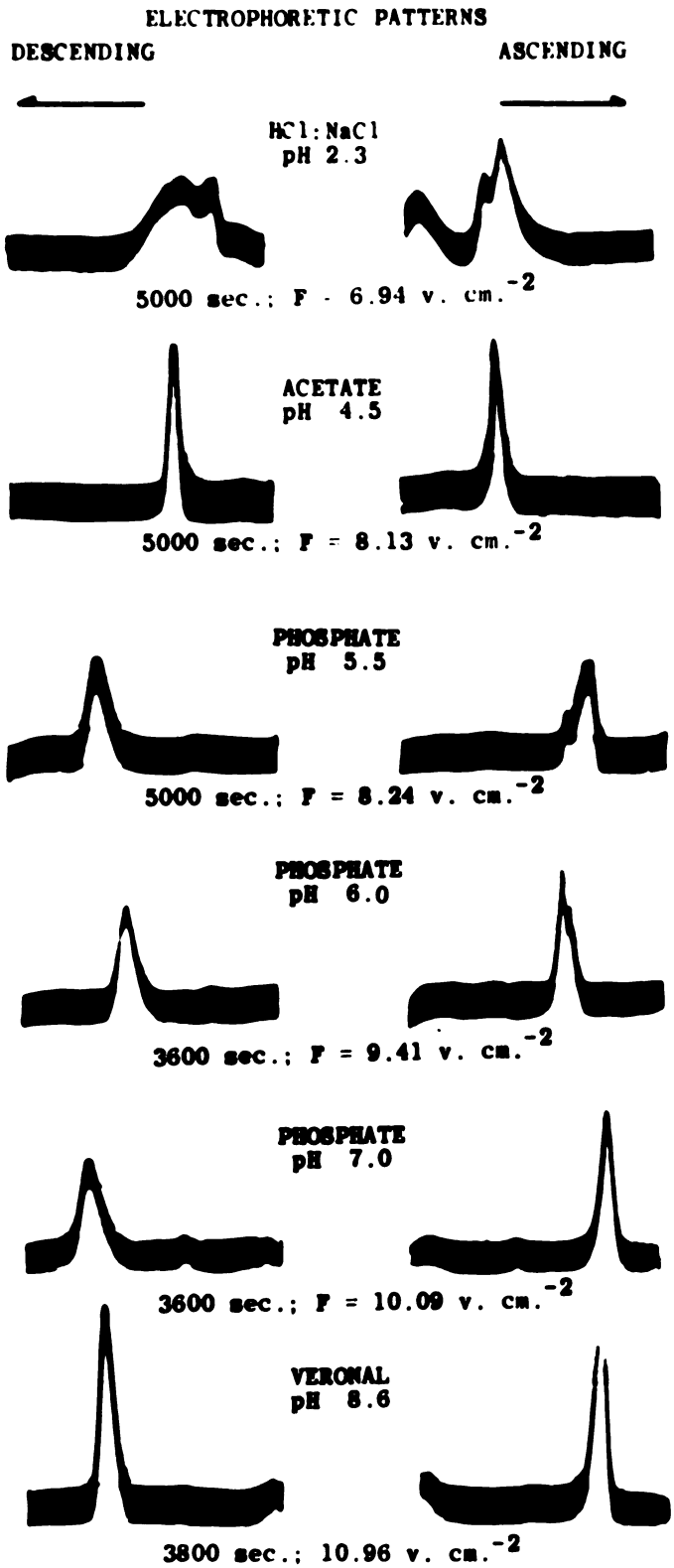

Figure 7. Electrophoretic patterns of k-casein Preparation No. 3 in buffers ranging from pH 2.3 to 8.6, $\Gamma/2 = 0.1$ (see Table 3).

Electrophoretic Mobility

Figure 8.
κ-casein P

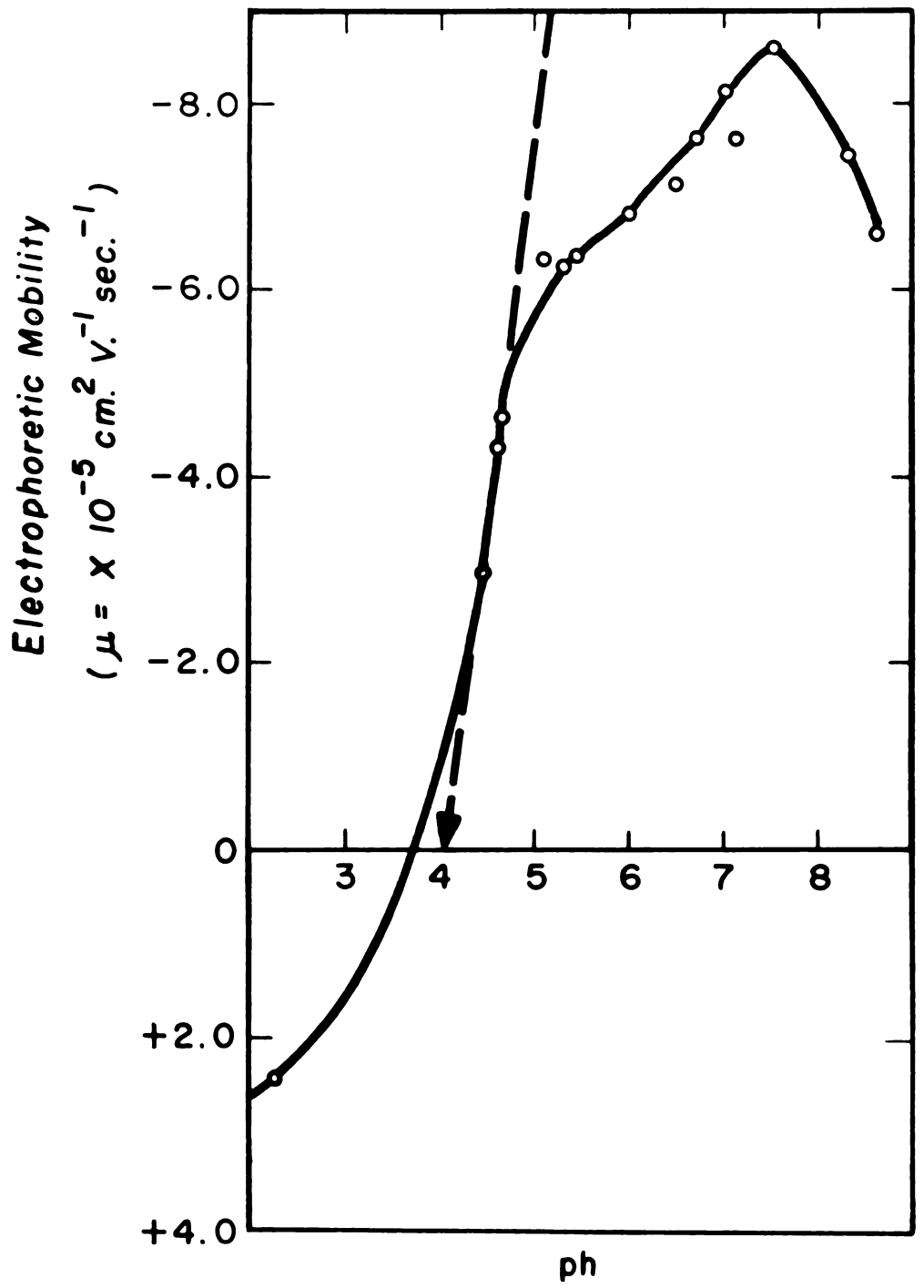

Figure 8. A plot of the descending electrophoretic mobilities of k-casein Preparation No. 3 at various pH values (see Table 3).

Figure 9.
Casein rem

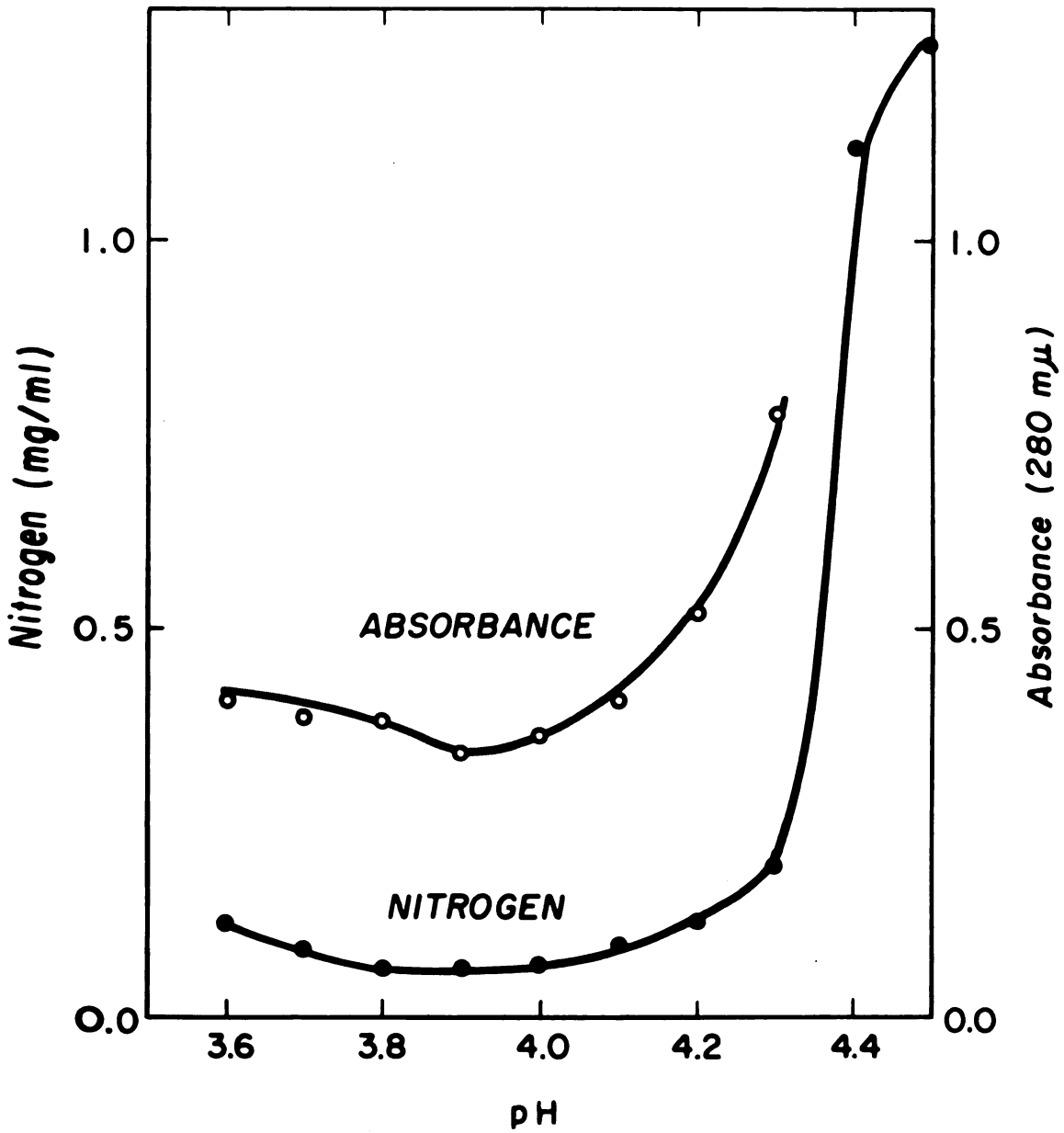

Figure 9. A plot of the nitrogen and 280 mμ absorption for the k-casein remaining in solution at pH values near the isoelectric point.

Figure 10
Preparati

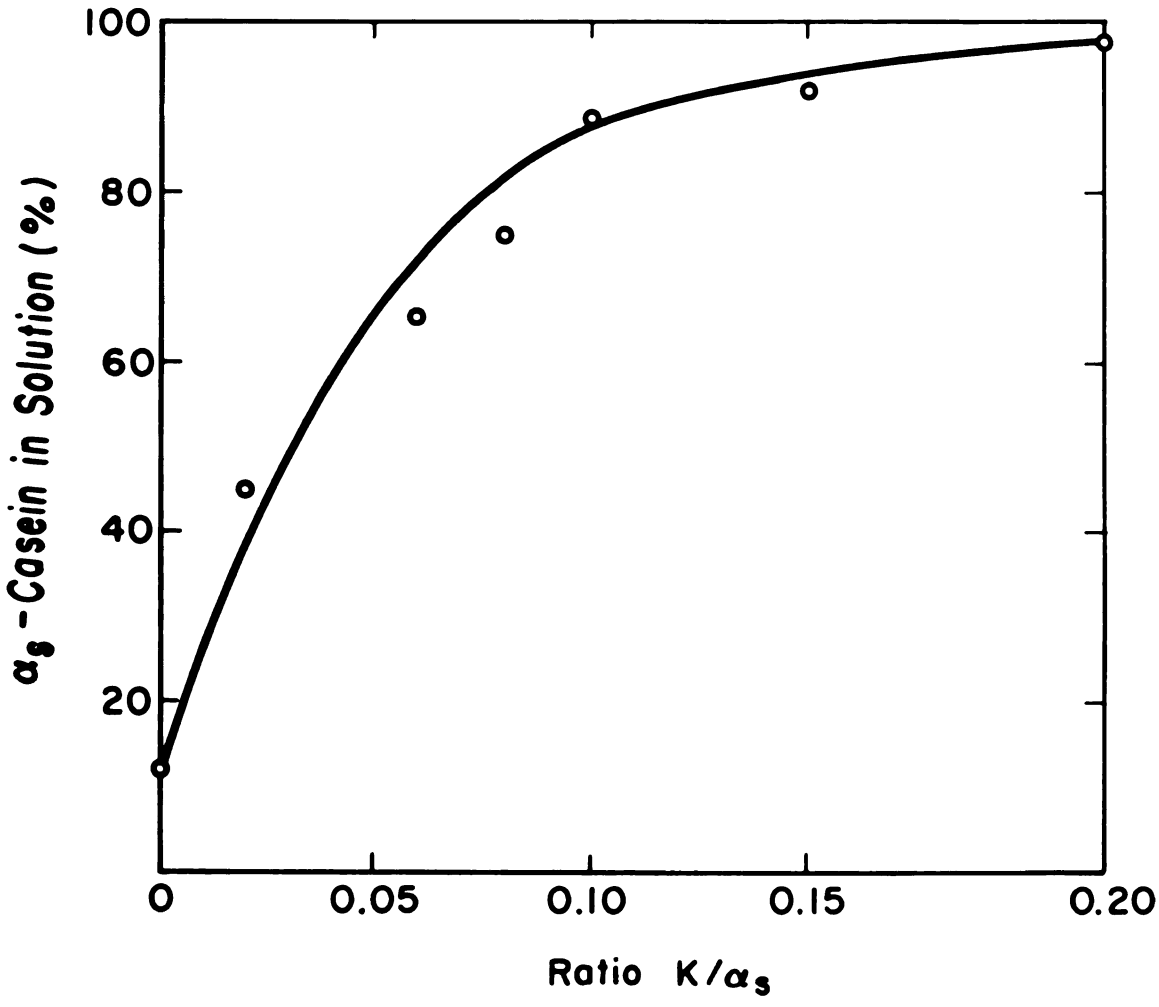

Figure 10. The stabilization of α_s-casein by various amounts of k-casein Preparation No. 3.

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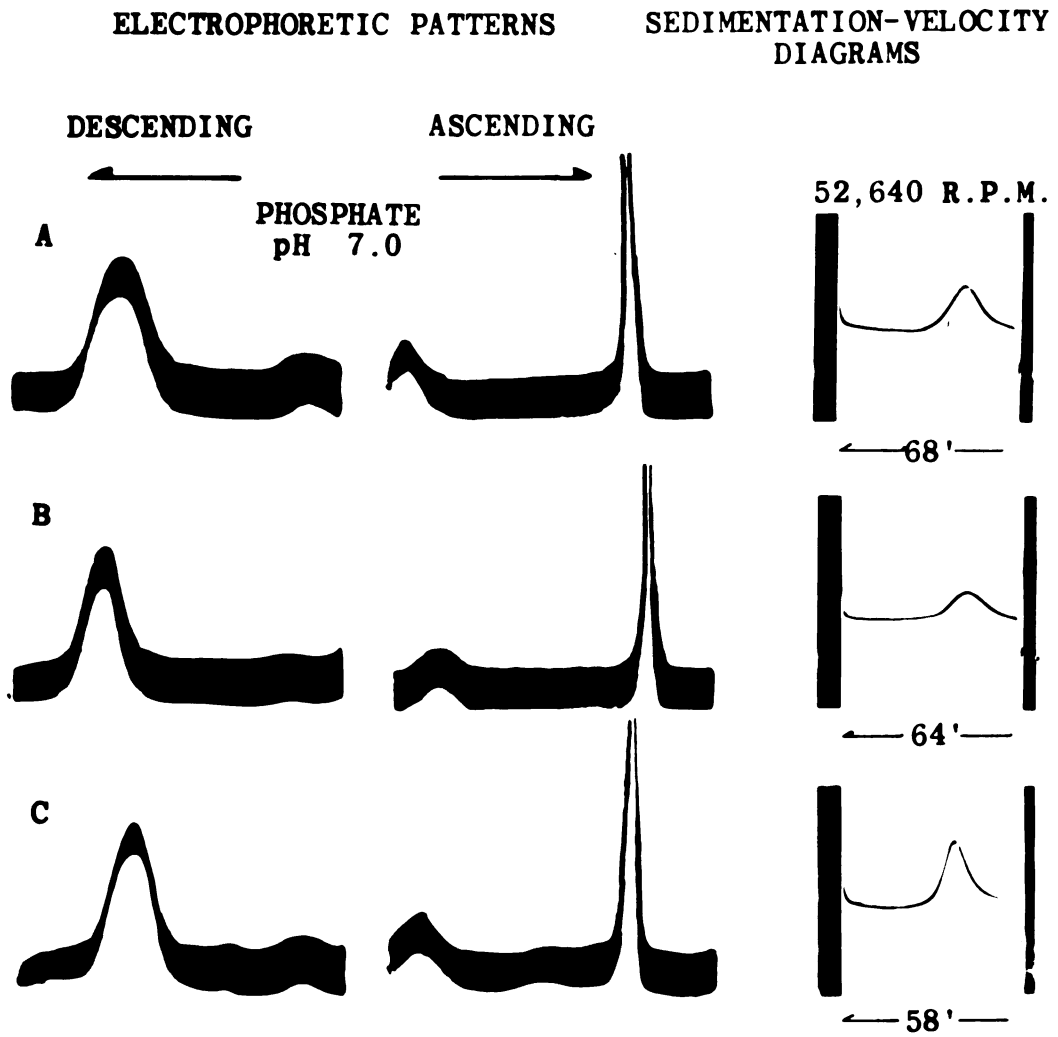

Figure 11. Electrophoretic patterns and sedimentation-velocity diagrams of A) Hipp et al. (1952) α-casein and k-/α_s-casein complexes induced by B) precipitation from 3.0 M urea and by C) momentarily adjusting a solution of the mixture to pH 12. Ultracentrifugation was conducted in phosphate buffer (pH 7.5) at 20° C.

DISCUSSION

General

The original goal was to obtain k-casein in good yields without requiring the use of a preparative ultracentrifuge. Large quantities of α_s- and β-casein could be removed from whole casein by adjusting solutions in 6.6 M urea to 12% TCA (w/w) at 3° C. The contaminating β-casein was difficult to remove, therefore, α-casein fraction prepared by a modification of the method of Hipp et al. (1952) was used as the starting material. The α_s-casein which remained with the k-casein following urea-TCA fractionation was easily removed by low speed centrifugation in the presence of 0.25 M $CaCl_2$. Cyanate ion was not operative in these concentrated urea solutions since isoelectric casein was used and the pH was lowered to < 4.7 when TCA was added. Marier and Rose (preprint), using a spectrophotometric method of improved sensitivity to detect cyanate ion, have shown that cyanate is not present in acidic urea solutions, ie., lower than pH 5.5.

k-Casein was obtained in quantities of 30 - 50% of the theoretical amount with a purity of 90 - 92%. Although the fraction did not have the desired purity for physical studies, it was relatively good compared to those preparations thus far reported. β-Casein (10%) was present in the k-casein prepared by Cheeseman (1962) and probably the λ-casein fraction was also a contaminate, although ultracentrifugal studies were not performed to detect this component (this writer has experienced greater difficulty in removing the λ-casein fraction than the β-casein). The method of Fox (1958) as modified by Morr (1959) gave a preparation which was apparently hemogeneous by free-boundary electrophoresis and sedimentation-

velocity, however, a synthetic boundary cell was not used for the sedimentation analyses. This method involved repeated exposures to high pH, several times in the presence of 6.6 M urea. Waugh (1958) reported a purification step for Fraction S which increased the purity to 90%. k-Casein prepared by the method of Long (1958) (also see Morr, 1959), from a mixture similar to Fraction S was shown to contain the slow-sedimenting λ-casein fraction in amounts of possibly more than 10%. Since the preparative ultracentrifuge was used initially for the constant pH methods, the volume of skimmilk was limited to one liter and thus the amount of k-casein obtained was also reduced. The method of McKenzie and Wake (1961) obtained good yields of apparently homogeneous k-casein, however, in some respects preparations by this method appeared to be "denatured" (Neelin, Rose and Tessier, 1962). Recently, Hill (1963) has published a method of preparing k-casein, but unfortunately the purity was not determined.

The ability to obtain relatively pure k-casein in large amounts was an asset when a preparative ultracentrifuge became available. Subsequent purification by ultracentrifugation and elution from G-75 Sephadex yielded a preparation which was 97% pure by analytical ultracentrifugation using a synthetic boundary cell. Similar purity was indicated by urea-starch gel electrophoresis which showed only small quantities of several leading bands. With respect to these two criteria of purity, this preparation appeared identical to that prepared by the McKenzie-Wake method.

Electrophoretic and Ultracentrifugal Characterization

The homogeneity of k-casein preparations was best determined by

sedimentation-velocity studies in phosphate buffer (pH 7, 0.1 $\Gamma/2$) at 4° C in a synthetic boundary cell. Free-boundary electrophoresis could detect β-casein, however, the λ-casein fraction moved with the k-casein (Long, 1958). At 4° C, both the β- and λ-casein appeared in the slow-sedimenting boundary since β-casein has an S_{20} of 1.36 S under these conditions (Sullivan, Fitzpatrick, Stanton, Annino, Kissel, and Palermiti, 1955). The observed sedimentation coefficients for k-casein were in accord with those reported by others (McKenzie and Wake, 1961; Waugh and von Hippel, 1956; Long, 1958; Morr, 1959).

Under certain conditions crude k-casein was observed to interact with β-casein. Free-boundary electrophoresis revealed a decrease in the relative area of the β-casein peak from 7.5% at pH 6.5 to 3.8% at pH 5.5. The relative area also decreased to 1% upon increasing the pH to 8.6. Furthermore, the mobility of k-casein reached a maximum at pH 7 to 7.5 and then decreased due to the incorporation of β-casein into the complex. Morr (1959) also observed a decrease in mobility at pH 8.4 as compared to 7. The work of McKenzie and Wake (1959a) on Fraction S showed 80% of the area attributable to k-casein by free-boundary electrophoresis, but only 50% of the area by ultracentrifugation. Also, the mobility at pH 7 (phosphate, 0.1 $\Gamma/2$) was -4.88 Tiselius units which is much lower than the value reported herein, again indicating interaction with β-casein. These researchers also studied the "α-casein split" using both whole acid casein and first-cycle casein. They found the split to occur more readily in acid casein and only when k-casein was present did it occur in other fractions, which led them to suggest that the split was due to the incorporation of k-casein into "difficultly reversible aggregates" with α_s-casein upon acid

precipitation. Their results are similar to the data reported here for the interaction of k- and β-casein in that the relative area of the β-casein peak decreased on lowering the pH from 7.8 to 6.3 -- the decrease being most marked at 6.3 -- and on raising the pH from 7.8 to 8.2 (however, the area was nearly as large at pH 8.8 as at 7.8). Also, the electrophoretic split in the pattern for crude k-casein at pH 5.5 and 6.0 reported here appeared similar to the data reported by McKenzie and Wake for whole casein.

Although these data are not in complete agreement, one must remember that k-casein occurs in whole casein as a complex with α_s-casein and therefore its properties will be influenced by the α_s-casein. On this basis, the "α-split" in whole casein may be due to an interaction of the k-, α_s-complex with β-casein. This view is supported by the fact that β-casein is known to be present in the boundary ascribed to the α-casein complex (Sullivan et al. 1955).

In alkaline buffers where k-casein is believed to be complexed with β-casein, the peak was not split, an observation attributed to the formation of the stable complexes. In acid buffers the casein complexes appeared to behave as association-dissociation systems resulting in the observed boundary split.

The isoelectric point of k-casein at 0.1 ionic strength lies between pH 3.8 and 4.1 as determined by electrophoretic mobility and minimum solubility studies. This value agrees with that reported by Hipp et al. (1961) for α_3-casein.

Stabilization and Interaction with α_s-Casein

The k-casein preparations were excellent stabilizers of α_s-casein.

At a k-/α-casein ratio of 1 : 10 about 90% of the α_s-casein was stabilized, a value similar to that reported by Zittle (1961).

Complex formation was induced in a mixture of 1 : 4 (w/w) k-/α_s-casein by dilution in concentrated urea solutions and by short exposure to pH 12. In both cases, electrophoretic and ultracentrifugal analysis obtained results similar to that for an α-casein fraction prepared by the method of Hipp et al. (1952). The electrophoretic mobility of the complex obtained from urea solutions was greater than that for the α-casein fraction or the other complex, however, in view of the preceding discussion of β-casein interaction, this increase could be explained on the basis of removal of β-casein from the complex. In fact, the electrophoretic pattern for the urea-formed complex showed less β-casein than that for the complex induced by pH adjustment. The sedimentation coefficient of the latter complex was larger than that for the α-casein fraction or the urea-formed complex, indicating either a different shape or a larger complex. One cannot conclude from these data that the complex which existed in the native micelle was formed, but only that a specific interaction occurred between k- and α_s-casein. Moreover, since these proteins were not completely pure, the role of β- and λ-caseins could not be determined.

Characteristics of the Preparation as a Primary Substrate for Rennin

Treatment of purified Preparations No. 3 and 3 A with rennin resulted in the formation of a precipitate in either redistilled water or buffered solution at pH 7. However, when a crude preparation containing large quantities (ca 30%) of λ-casein fraction was treated with rennin in redistilled water, a precipitate did not form. Addition of NaCl to 0.1

M concentration caused the solution to become opalescent, but still no precipitation occurred. Possibly an interaction had occurred between k-casein and the λ-casein fraction resulting in greater stability of the para-k-casein. Long (1958) also observed an interaction between k- and λ-casein. After repeated preparative centrifugation of crude k-casein in 0.2 M NaCl, analytical ultracentrifugation showed only a small amount of λ-casein. However, a similar analysis conducted 48 hours later revealed a considerable amount of λ-casein. Crude k-caseins, containing the λ-casein fraction, served as good stabilizers of α_s-casein, but studies were not performed to compare these with purer preparations. Nevertheless, the possibility exists that an interaction of the λ-casein fraction with k-casein may increase the latter's ability to stabilize α_s-casein.

The soluble peptides cleaved from k-casein by the action of rennin in redistilled water at approximately neutral pH were obtained by ultracentrifugation of the precipitated para-k-casein which yielded a hard pellet, easily separated from the supernatant. Not all of the soluble peptides, consisting of 30% by weight of the k-casein, were soluble in 12% TCA solution, as also evidenced by the amount of soluble nitrogen compared to the TCA-soluble nitrogen. Twenty-three per cent of the nitrogen was classified as soluble, a value in agreement with that reported by Wake (1959). The unpurified Preparation No. 3 A yielded a 7% increase in the TCA-soluble nitrogen. Probably this would have been greater for the purified material (Wake, 1959, reported 6.7% and Beeby, 1963, reported 8 - 9%). These results seem to indicate that more than one peptide is released by rennin. However, according to Wake (1959) some of the glyco-macropeptide -- 12% TCA-soluble material -- may be precipitated by 12% TCA.

A molecular weight of 8400 was calculated for the peptides using the sub-unit molecular weight of k-casein (see Part II of this thesis) and the fact that 30% of the original k-casein remained after removal of para-k-casein. This value was similar to the value obtained by short-column equilibrium -- ca 6000 -- and the range of values -- 6000 to 8000 -- reported by Nitschmann et al. (1957). Sedimentation-equilibrium experiments performed in this laboratory have indicated a molecular weight of ca 9000 for the glyco-macropeptide (McCabe, unpublished data).

Other Proteins Obtained During the Preparation of k-Casein or as a Result of a Slight Alteration in the Preparative Procedure

By using α-casein fraction, free of electrophoretically discernible β-casein, as the starting material, α_s-casein was obtained as a precipitate from 6.6 M urea -- 12% TCA solutions. The preparation was homogeneous when examined by free-boundary electrophoresis and sedimentation-velocity ultracentrifugation. However, a band which appeared to move slightly ahead of β-casein was observed in urea-starch gel electrophoretic patterns (Figure 5). Nevertheless, α_s-caseins prepared by other procedures have shown greater heterogeneity, e.g., Neelin et al. (1962), when examined by this method. The value obtained for the sedimentation coefficient was in accord with those reported by Waugh and von Hippel (1956), Long (1958), and McKenzie and Wake (1959b); namely, 4.5 S, 4.6 S and 4.4 S, respectively. Also the electrophoretic mobility agreed with that given by McKenzie and Wake (1959a); $\mu = -7.17$ Tiselius units in veronal buffer, pH 8.2.

Incorporation of 10.6 g/l NaCl into the 6.6 M urea used to purify the α-casein fraction resulted in the complete absence of k-casein in the

material obtained in Step 6 of the preparation scheme (Figure 1). On the basis of the stability to calcium ion, sedimentation coefficient, non-reactivity with rennin, and inability to stabilize α_s-casein, the material was considered similar to the λ-casein fraction reported by Long (1958). There seems to be some inconsistancy in the product obtained (Neelin et al., 1962). Three of the four preparations made in this laboratory were essentially identical. The fraction appears homogeneous in neutral phosphate buffer, but ultracentrifugal studies in 5 M guanidine · HCl clearly indicated molecular heterogeneity. Urea-starch gel electrophoresis also revealed numerous bands (Figure 5), all of which moved ahead of the β-casein position. Particularly characteristic of the λ-casein fraction was the band which moved with the front. Whether or not the band in the α_s-casein region was α_s-casein remains to be determined. Since the fraction is homogeneous in non-dissociating solvents, a complex must exist between the constituent proteins under the conditions employed.

SUMMARY OF PART I

A method was developed for preparing k-casein of about 90% purity in yields approximating 30 - 50% of the amount theoretically present. The preparation was characterized by free-boundary electrophoresis, urea-starch gel electrophoresis, sedimentation-velocity ultracentrifugation, ability to stabilize α_s-casein, and its reaction with rennin. The results of these characterizations confirmed that the protein possessed properties typical of k-casein as reported previously. Although the preparation was not as homogeneous as desired for physical studies, the method provided large quantities of the crude k-casein which could be readily purified, yielding a final product of 97% purity.

The preparation scheme also afforded means of obtaining the α_s- and λ-casein fractions.

PART II

PROPERTIES OF KAPPA-CASEIN

INTRODUCTION TO PART II

The k-casein isolated from bovine milk forms relatively uniform aggregates in inorganic salt solutions below pH 11. To assess the properties of this protein more completely, the associated protein molecules must be dissociated prior to characterization. Unfortunately, the dissociation can be accomplished only by using strong dissociating solvents such as concentrated acetic acid, guanidine, urea or sodium dodecyl sulfate. These agents make the determination of thermodynamic or hydrodynamic properties less accurate due to preferential interactions and the non-ideal behavior of protein in their presence.

However, studies in recent years have shown that these errors probably were not greatly significant. To cite a few of the many examples; a) Harrup and Woods (1961) found that molecular weights of bovine serum albumin, egg albumin, β-lactoglobulin, lysozyme and insulin in anhydrous formic acid were in agreement with those determined in other solvents; b) Kielley and Harrington (1960) found that the molecular weight of ribonuclease in 5 M guanidine · HCl was in accord with the known value; c) Trautman and Crampton (1959) obtained the same result for ribonuclease in 6 M urea; d) Massey, Hofmann, and Palmer (1962) obtained an Archibald molecular weight for lipoyl dehydrogenase in 6.5 M urea which agreed with the chemical analyses of amino acids and FAD; e) Hofmann and Harrison (1963) found that the Archibald molecular weight of apoferritin in SDS solutions was in accord with x-ray diffraction studies; f) Yphantis and Waugh (1957) obtained a molecular weight for insulin in 33% acetic acid — 0.15 M NaCl which agreed with the chemical analysis; and g) Criddle, Bock, Green, and Tisdale (1962) obtained the same molecular weight for the structural

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protein of mitochondrion in 67% acetic acid, 8 M urea, and 0.1% SDS.

Information relating to the chemical composition and physical characterization of k-casein in neutral salt solutions and in the presence of dissociating agents were the objectives of the experiments reported in this section of the thesis.

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EXPERIMENTAL

Apparatus

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 for most of the velocity studies and also to determine the initial concentrations in terms of optical areas. Equilibrium experiments were performed in a double-sector cell. Both of these centerpieces were the filled-Epon type. For some of the velocity studies a single sector, analytical cell with a Kel-F plastic centerpiece was used. The 12 mm cells equipped with quartz windows were used in all determinations. Centrifugation was performed in an An-D duralumin rotor and a General Electric AH-6 mercury lamp served as the light source.

The schlieren patterns were preserved on Kodak metallographic glass plates and measured with a Nikon Model 6 Shadowgraph microcomparator. The micrometer stage traveled 25 mm on the y-axis, 50 mm on the x-axis and was read directly to 0.002 mm.

Viscosities were determined with a Cannon-Ubbelohde semi-micro dilution viscometer which could be operated with a volume of solution ranging from 1 to 20 ml. A circulating, Precision Scientific water bath equipped with a Precision micro-set thermoregulator was used to control the temperature for viscosity and density determinations. The temperature was controlled to $\pm$ 0.01° C as indicated by a Philadelphia differential thermometer.

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A Starrett microsyringe-burette was used for sulfhydryl group titrations and to fill the ultracentrifuge cell for short-column equilibrium experiments. The microsyringe had a total capacity of 250 µl and the micrometer could be read to 0.1 µl.

Spectrophotometric measurements were made with a Beckman DK-2 ratio-recording spectrophotometer or a Beckman DU spectrophotometer using quartz cells with a 1 cm light path.

The hydrogen ion concentrations of various buffers and solutions were determined with a Beckman Model G pH meter using a glass electrode.

Materials were dried in temperature controlled Cenco vacuum oven.

Chemicals

p-Mercuribenzoate (PMB) was obtained from the Sigma Chemical Company. Glutathione (GSH) was purchased from Matheson Company. Guanidine • HCl (GU) and 2-mercaptoethanol (ME) were Eastman Organic Chemical 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 below 60° C in the vacuum oven. Sodium dodecyl sulfate (SDS) was obtained from Fisher Scientific and used without further purification. Fluorocarbon oil FC-43, purchased from Spinco, was used to form a false bottom for ultracentrifugal studies. N-acetylneuraminic acid (NANA), used to obtain a standard curve for NANA determination, was supplied by General Biochemicals.

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Unless otherwise specified, all chemicals were reagent grade quality.

Chemical Methods

Amino Acid Analysis[1]

The amino acid analyses were performed on 24, 48, and 72 hour acid hydrolysate, using a Beckman Acid Analyzer. The values obtained were extrapolated to zero time for acids whose values decreased with time of hydrolysis. The quantity of protein analyzed was determined from a micro-Kjeldahl nitrogen analysis. Percentage of nitrogen in the protein was determined on samples dried at 105° C over P_2O_5 in the vacuum oven for 48 hours.

Nitrogen

The digestion mixture consisted of 5 g SeO_2 and 5 g $CuSO_4 \cdot 5H_2O$ in 500 ml of concentrated H_2SO_4. Protein (1-5mg) was digested with 1 ml of digestion mixture on an electric burner for 4 hours, then 5 drops of 30% H_2O_2 was added and digestion continued for another half hour. After cooling, 2 ml of redistilled water was added. Immediately before attaching the digestion flask to the distillation unit, the solution was neutralized with ca 9 ml of 40% KOH solution. The ammonia was distilled by heating with a microburner and collected in 5 ml of a 4% boric acid solution containing 3 drops of indicator. Standard 0.1038 N HCl diluted 5 times was used to titrate the ammonia. The normality of the standard was determined by titration with Sigma 121, tris (hydroxymethyl) aminomethane.

[1] The amino acid analysis was obtained through the courtesy of Drs. J. P. Riehm, J. C. Speck and H. A. Lillevik of the Biochemistry Department.

The recovery of nitrogen from standards was 100.2 ± 2.9% using ammonium sulfate and 100.0 ± 3.3% using tryptophan. The per cent nitrogen in β-lactoglobulin determined in quadruplicate on ca 5 mg samples was 15.63 ± .03% as compared to a reported values of 15.60% (McMeekin, 1954).

Sulfur

The total sulfur was determined by Spang Microanalytical Laboratory, Ann Arbor, Michigan. Prior to analysis, the protein was dried over P_2O_5 at 105° C for 48 hours in a vacuum oven.

Phosphorus

Phosphorus was determined colorimetrically in a wet digest (H_2SO_4 and H_2O_2) of the protein -- dried in the vacuum oven over P_2O_5 and weighed with the microbalance -- by the method of Sumner (1944). Ferrous sulfate was added in slight excess to reduce the phosphomolybdic acid to a blue color which was read at 610 mμ.

N-Acetylneuraminic Acid

The method used was reported by Aminoff (1961). Similar to the procedure given by Warren (1959), the method is also specific for the free acid. The protein was dried to constant weight in the vacuum oven over P_2O_5 at 105° C and weighed with the microbalance. A standard curve was prepared using commerically available NANA. An amount of protein corresponding to 20 - 40 μg of NANA was dissolved in 0.5 ml of 0.1 N H_2SO_4 and hydrolyzed for 1 hour at 80° C thus releasing the NANA. Periodate reagent was added and the sample incubated at 37° C for 30 min, after which the excess periodate was reduced with sodium aresnite.

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Thiobarbituric acid reagent was added and the color developed by heating in a boiling water bath for 7.5 min. The color compound was extracted in 5 ml of acid-butanol and the absorbancy at 549 mμ determined in a Beckman DU spectrophotometer.

Disulfide Bond Reduction

The disulfide bonds of k-casein were reduced with mercaptoethanol (ME) at a level of ca 2 μl/mg of protein in 5 M guanidine . HCl (GU) adjusted to pH 8.4 with methylamine (Anfinsen and Haber 1961). Following a reduction period of about 24 hours, the solution was adjusted to pH 3 with glacial acetic acid. The reducing agent and GU were removed by passing the solution over G-25 Sephadex equilibrated with 0.1 M acetic acid. Complete elution of the protein was achieved approximately 40 to 50 ml prior to the elution of ME. Reoxidation of the sulfhydryl groups should be negligible under the acidic conditions of this elution.

Sulfhydryl Group Titration

The sulfhydryl groups were titrated essentially according to the procedure described by Boyer (1954) and reviewed by Benesch and Benesch (1962). However, to prevent reoxidation of the reduced disulfide bonds following the removal of ME, the following procedure was devised: a) a protein determination was made on an aliquot of the protein solution immediately following its elution in 0.1 M acetic acid from the Sephadex G-25 columns, b) excess PMB was added directly to the reduced protein in 0.1 M acetic acid, and c) the pH was adjusted to 7 with 0.1 N NaOH.

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The volume of protein solution was accurately measured after removing the aliquot and before adding the PMB solution. The concentration was determined on the aliquot by measuring the absorbancy at 280 mμ. The amount of portein in the reaction mixture was usually 15 - 20 mg.

A known quantity of PMB dissolved in redistilled water was added to the reduced k-casein and amounted to approximately ten times the quantity required to react with the newly formed SH groups. The concentration of PMB in solution was determined just prior to addition by titration with a freshly prepared standard glutathione (GSH) solution (Benesch and Benesch, 1962). The concentration of the standard GSH solution was determined by weighing the desiccated crystals on the microbalance and dissolving with water in a volumetric flask. This method provided results which were in good agreement with the concentration calculated by using the extinction coefficient at 232 mμ given by Boyer (1954).

Fifty milliliters of protein solution were quantitatively transferred to a 100 ml volumetric flask. After standing for 1 hour at pH 7, the solution was made 7 M with respect to urea to insure complete reaction of the SH groups and the solution made to volume. The unreacted PMB in aliquots of this solution was titrated with the standard GSH solution used previously to titrate the PMB solution. Thus, standard GSH solution was added to both the sample and reference cell in equal volumes with a Starrett microsyringe and the end point determined by following the change in absorption at 250 mμ. A blank consisting of a volume of 0.1 M acetic acid was treated in exactly the same manner as the protein solution. The difference between the amount of PMB in the blank and the sample represented the quantity of PMB bound to the protein.

One determination of the number of SH groups was made by a direct titration with standard PMB solution. In this experiment, however, a clear solution was not obtained unless the pH was adjusted to 9. Some re-oxidation could occur under these conditions.

Nitroprusside

The test was conducted in the presence of 5 M guanidine · HCl according to the procedure described by Hamilton (1960). One part of sodium nitroprusside to two parts of sodium carbonate were ground in a mortar until no crystals of the former could be distinguished. About 10 mg of this mixture was added to 1 ml of the protein solution after displacing the air with nitrogen. The test was performed on a reagent blank and a sample of β-lactoglobulin as a positive control concurrently with each experiment.

Physical Methods

Ultracentrifugation

Determination of the protein concentration. The freeze-dried protein was weighed on a microbalance and dissolved in an appropriate solvent. The weight was corrected for the moisture content as determined on a portion of the freeze-dried sample by drying to constant weight in the vacuum oven at 105° C over P_2O_5. Following dialysis against the solvent, the solution volume was measured with a Hamilton Microliter syringe. In some instances the concentration was rechecked by nitrogen analysis.

In several cases, the concentration was determined by measuring the area of the schlieren pattern for the initial boundary formed in

synthetic boundary cell. The amount of protein was determined from a standard plot of area and concentration constructed from data obtained in a similar manner.

Sedimentation-equilibrium. The short-column equilibrium technique described by Van Holde and Baldwin (1958) was used. Centrifugations were performed in the double sector cell at 25.0° C using solution column lengths of 1 to 2 mm. A period of 24 hours was allowed for attainment of equilibrium. This time was more than adequate as indicated by characteristics of the schlieren patterns and calculations made from an assumed, approximate diffusion coefficient.

Schlieren patterns for the concentrated urea and GU solvents may also exhibit some curvature even at the low speed used. Consequently, the column which contained the protein solution was bracketed by the solvent column, ie., the false bottom (flourocarbon oil, FC-43) of the solvent column was slightly below and the air-liquid meniscus slightly above that for the solution column. To achieve this physical relationship the amount of solution required to produce the desired column length was calculated and precisely measured into the cell with a Starrett microsyringe along with predetermined quantities of flourocarbon oil and solvent. Normally, the amounts added were a) 110 µl of oil and 70 µl of solution in the solution sector and b) 100 µl of oil and 90 µl of solvent in the solvent sector, resulting in a 10 µl differential on each side of the solution column.

The protein solutions were dialyzed against their respective solvents for approximately one week at 20° C prior to sedimentation analyses. Optical areas obtained with the synthetic boundary cell were used as a measure of the concentration for molecular weight calculations. Details of the procedure are presented in Appendix II.

Diffusion coefficients were calculated from patterns obtained during the approach to equilibrium experiments according to the method of Sophianopoulos, Rhodes, Holcomb, and Van Holde (1962).

Approach-to-Equilibrium. This procedure was used principally to determine the molecular weight of the low-molecular weight component in a heterogeneous system. Experiments were performed at various rotor speeds and the data plotted as suggested by Trautman (1956) and adapted to heterogeneous systems by Erlander and Foster (1959). The details of this procedure are given in Appendix II. Since the concentrated GU, urea, and SDS solvents formed a gradient under these conditions, precaution was taken to assure that exactly the same volumes of solution and solvent were placed in the double sector cell. The patterns thus obtained showed only one liquid-air meniscus.

In some trials, only one rotor speed was used in which case the weight-average molecular weight was calculated as described by Schachman (1957).

Sedimentation-velocity. These experiments were performed at 20 or 25° C using a rotor speed of 59,780 RPM. The low sedimentation coefficients observed for the proteins in the presence of the dissociating solvents precluded the use of the synthetic boundary cell in these studies. Accurate sedimentation coefficients were obtained with this cell for coefficients less than 1 S (Schachman, 1959) and the problem of restricted diffusion at the meniscus was eliminated. Furthermore, concentrated solutions of GU, urea, and SDS also form gradients at the meniscus making observations of the protein boundary difficult unless a synthetic boundary cell is used. Then, the protein boundary is formed at the start of the experiment and at a considerable distance from the

air-liquid meniscus. The cell was filled by placing 300 µl of protein solution in the lower compartment and 100 µl of solvent, which had been dialyzed against the solution, in the upper compartment.

Sedimentation coefficients, determined by plotting the logarithm of the maximum ordinate against time, were corrected to values corresponding to water at 20° C ($S_{20,w}$). The concentration corresponding to a particular $S_{20,w}$ was the average concentration of the first and last frame used to obtain the $S_{20,}$ and was corrected for radial dilution according to Schachman (1959). Detailed calculations are presented in Appendix II.

Densities and partial specific volume. The solvent densities were determined with 25 ml pycnometers at 25.0 ± 0.01° C. Solution densities were calculated according to Fujita (1962) using the relation

$$\rho \text{ solution} = \rho \text{ solvent} + (1 - \bar{v} \rho \text{ solvent}) \, c$$

where $\bar{v}$ is the partial specific volume of the protein and c its concentration in g/ml. Freshly boiled, redistilled water was used to calibrate the pycnometers before each determination. Duplicate determinations agreed to ± 0.0001 or better.

The partial specific volume was calculated from the amino acid analysis of the protein using the relation

$$\bar{v} = \frac{\sum_i v_i \, W_i}{\sum_i W_i}$$

where $\bar{v}$ is the partial specific volume of the protein, v_i the specific volume of the i^{th} amino acid residue, and W_i the weight per cent of the

i^{th} amino acid residue (Cohn and Edsall, 1943; McMeekin, Groves, and Hipp, 1949). The weight per cent of the amino acid residues is given by

$$W_i = g_i/100g \text{ protein} \left(1 - \frac{18}{M_i}\right)$$

where g_i is the grams of the i^{th} amino acid and M_i the molecular weight of this amino acid.

Intrinsic viscosity determinations. The relative viscosities were determined at 25.0 ± 0.01° C. The outflow of time for water was 285.8 seconds. Four to six observations of the outflow time were obtained for each determination. All solutions were filtered through a sintered-glass filter. The relative viscosity was calculated according to the relation

$$\eta/\eta_o = \frac{t}{t_o} \frac{\rho}{\rho_o}$$

where η, t, ρ are the viscosity, outflow time, and solution density and η_o, t_o, and ρ_o represent similar quantities for the solvent. The solution density was calculated as previously described. The intrinsic viscosity $[\eta]$ was determined by plotting the reduced viscosity (η_{sp}/c) against the protein concentration in g/ml, where $\eta_{sp} = \eta/\eta_o - 1$.

RESULTS

Composition

The amino acid composition of k-casein is shown in Table 4. These values were based on a nitrogen content of 15.3% as determined by micro-Kjeldahl analysis. The values for Thr, Ser and NH_3 were obtained by extrapolation to zero hydrolysis time. The weight per cent of the amino acid residues (W_i) are presented together with a comparison of the number of residues per 28,000 g of protein for a) purified Preparation No. 3A, b) the k-casein studied by Jollès *et al.* (1962), and c) α_3-casein reported by Hipp *et al.* (1961). The residues were calculated from their data which were reported as g/100 g protein. A summation of the residue weights which includes in addition to the amino acid residues, the values for NANA and phosphorus, as well as the values of Jollès *et al.* (1962) for Cys, Try, galactose, and galactosamine amounts to 95 per cent.

The partial specific volume was calculated from the amino acid residue weights and the concentration of NANA (1.4%) for the purified Preparation No. 3A. The weight percentages for Cys (1.30%), Try (0.96%), galactose (1.4%), and galactosamine (1.2%) were reported by Jollès *et al.* (1962). By this method a $\bar{v}$ of 0.729 was obtained,

e.g., $$\bar{v} = \frac{\sum_i v_i W_i}{\sum_i W_i} = \frac{68.925}{94.55} = 0.729.$$

Partial specific volumes of 0.62 for hexoses and hexosamines and 0.59 for sialic acid were used (Bezkorovainy and Doherty, 1962).

The average of several determinations gave values of 0.22% phosphorus and 1.4% NANA for purified Preparation No. 3A. The percentage

of P reported by Jollès et al. (1962) and Thompson and Pepper (1962) was in good agreement with this value (0.217 and 0.22, respectively). The values reported for NANA have been subject to considerable variation depending upon the method of preparation (Marier, Tessier, and Rose, 1963). The purified k-casein preparation (No. 3A) contained 0.70 ± 0.02% sulfur. The calculated molecular weights in terms of the percentages of NANA, Try, P, and S are shown in Table 5.

Physical Properties of k-Casein

Properties in Non-Dissociating Solvents

The results of sedimentation-velocity studies performed on different lots of purified No. 3A at temperatures of 3° and 25° C and ionic strengths from 0.1 to 0.5 are shown in Table 6. The S_{20} value for a McKenzie-Wake preparation has also been included. Temperature and ionic strength had no significant effect on the sedimentation coefficient. The data were fitted to the equation $S_{20} = S^{\circ}_{20}$ (1-kc) giving the result

$$S_{20} = 15.6\ (1 - 0.0165C) \pm 0.25,$$

where S_{20} is in Svedbergs and c in mg/ml.

The viscosity of this preparation was studied in 0.1 M NaCl at 25.0° C. Fitting the data to the Huggins equation (Tanford, 1961) gave

$$\eta_{sp/c} = 9.5 + (0.217)\ (90.25)\ c \pm 0.06,$$

where the intrinsic viscosity was 9.5, the Huggins constant 0.217, and c was in mg/ml. The data are plotted in curve C of Figure 12.

Properties in Dissociating Solvents

Sedimentation characteristics in SDS solutions. k-Casein

(purified No. 3A) was sedimented in buffers at pH 8.6 or 9.25 which contained from 2.5 to 40 mg/ml SDS. Usually the solutions were placed in the regular analytical cell without prior dialysis. Thus, a boundary due to SDS did not appear until the unbound SDS concentration exceeded the critical micelle concentration, i.e., 0.11% (Hofman and Harrison, 1963). An SDS boundary became apparent as the weight-ratio of SDS/protein approached 1. Apparently 0.5 to 1 g SDS was bound per gram of protein. Since the SDS and protein boundary were not completely separated, the pattern was measured on the Trautman z-scale and $X^2 \Delta Y$ plotted against X to correct for radial dilution. Gaussian curves were fitted to these plots to obtain the maximum ordinate for each peak. The calculated sedimentation coefficients are recorded in Table 7.

A value of approximately 2.3 S was obtained for the sedimentation coefficient in SDS concentrations ranging from 2.5 to 20 mg/ml. Archibald molecular weights were obtained for k-casein solutions in veronal buffer containing 10 mg/ml SDS and 5 and 10 mg/ml of protein. The solvent, dialyzed against the protein solution, was used as a reference. The apparent molecular weights were corrected according to the equation

$$M = M^{app} \Big/ 1 + x_1 \frac{(1 - \bar{v}_1 \rho)}{(1 - \bar{v}_p \rho)},$$

where x_1 represents the grams of SDS bound by one gram of protein, $\bar{v}_1$ the partial specific volume of SDS, $\bar{v}_p$ the partial specific volume of the protein, and ρ the solution density (Schachman, 1960). Even at a maximum x_1, i.e. 1.0, molecular weights were calculated to be approximately 100,000. The sedimentation coefficient decreased as the

SDS concentration was increased to 30 or 40 mg/ml. This characteristic could be caused by unfolding or swelling as well as by molecular dissociation. Furthermore, the steep refractive index gradients due to free SDS, and the charge effect caused by bound SDS at these high concentrations make molecular weight calculations of questionable reliability. Consequently, further studies along those lines were not performed.

Sedimentation and viscosity characteristics in 5.0 M GU solutions. The results of short-column equilibrium experiments performed on k-casein (purified No. 3A) in 5.0 M GU at pH 4.8 are recorded in Table 8 and Figure 13 (filled circles). A molecular weight of 126,000 was obtained by a least-squares extrapolation to zero concentration. A marked concentration dependence of the apparent weight-average molecular weight was noted. The ratio M_z/M_w was greater at a concentration of 3 mg/ml. Additionally, a Van Holde-Baldwin plot of the data showed considerable deviation from linearity (Figure 14B).

The molecular weight of the small component present in a 3 mg/ml solution of k-casein in 5.0 M GU was determined from a Trautman plot of approach-to-equilibrium data (see Figure 15) and yielded a value of 54,000.

Sedimentation-velocity data were best represented by the linear relation

$$S_{20,w} = 3.18\,(1-0.0178\,c),$$

where $S_{20,w}$ is in Svedbergs and c in mg/ml. A value of 3.1 Ficks $-D_{20,w}-$ was calculated from a short-column equilibrium experiment at a protein concentration of 10.3 mg/ml. An approximation of the

molecular weight was obtained by combining this value with the sedimentation coefficient determined at the same concentration. This method assumes that the sedimentation and diffusion coefficients have the same concentration dependence. Nevertheless, an apparent molecular weight of 75,000 was obtained which was in agreement with the equilibrium data determined at the concentration. An intrinsic viscosity of 31 ml/g was determined from an evaluation of a reduced viscosity plot, see Figure 12B. The Scherage-Mandelkern constant, β, calculated from these data was 2.00×10^6. The data are summarized in Table 9.

Sedimentation and viscosity characteristics in concentrated acetic acid solutions adjusted to 0.15 M NaCl. The short-column equilibrium data obtained in 67% acetic acid are represented by the open circles in Figure 13, and compared with the values for reduced k-casein, Table 10. The apparent weight-average molecular weights, which were strongly concentration dependent, yielded a value of 124,000 at zero concentration by least-squares extrapolation. Similar values were obtained from an experiment performed on purified Fraction S. Ratios for the relationship M_z/M_w were nearly one in all cases. Moreover, the Van Holde-Baldwin plots were linear for these data, see Figure 14A.

In 33% acetic acid, however, k-casein appeared to be polydispersed. A Trautman plot of approach-to-equilibrium data for a 6.8 mg/ml solution is shown in Figure 16. The molecular weight of the small component evaluated from the final slope was 58,000.

Selected hydrodynamic properties were determined in 67% acetic acid and are summarized in Table 9. The sedimentation coefficients

were fitted to the equation

$$S_{20,w} = 2.7\ (1 - 0.0302\ c)$$

where $S_{20,w}$ is in Svedbergs and c in mg/ml. A value of 2.4 Ficks was obtained for the diffusion coefficient at zero concentration by extrapolation of values calculated from the short-column equilibrium experiments. At a concentration of 7.5 mg/ml, the value was 1.5 Ficks. Substitution of these data into the Svedberg equation yielded a molecular weight of 101,000 which is in relatively good agreement with the value obtained by the short-column equilibrium technique.

The intrinsic viscosity was found to be 35 ml/g as determined from a reduced viscosity plot (Figure 12A). A value for β of 1.8×10^6 was calculated.

Sedimentation characteristics in 7.0 M urea solutions at pH 8.5. k-Casein (purified No. 3A) was polydispersed in this solvent as indicated by the results obtained from a short-column equilibrium experiment. At a protein concentration of 5 mg/ml the M_w and M_z were 108,000 and 213,000, respectively, giving a ratio, M_z/M_w of 1.97. Approach-to-equilibrium studies of this solution resulted in values of 57,000, 118,000, and 144,000 for the molecular weights of the small component, M_w, and M_z, respectively. A Trautman plot of the data is shown in Figure 17.

Sedimentation and viscosity characteristics in pH 12.2 phosphate buffer. Archibald molecular weights (M_w), calculated from the meniscus patterns, were averaged to approximately 24,000 for a 10 mg/ml solution of purified Preparation No. 3. A molecular weight of 23,400 was obtained from a Trautman plot of approach-to-equilibrium data for a

5.3 mg/ml solution of purified Preparation No. 3A. Unlike k-casein in 7.0 M urea, 5.0 M GU, and 33% acetic acid, the Trautman plot for these data fitted a straight line, see Figure 18.

The diffusion coefficient for a protein concentration of 8 mg/ml was 5.8 Ficks, obtained in the Tiselius electrophoresis cell by the height-area method. The sedimentation coefficients fitted the equation

$$S_{20,w} = 1.4\ (1 - 0.0172\ c)$$

where $S_{20,w}$ is in Svedbergs and c in mg/ml. A molecular weight of 22,300 was obtained by inserting these data into the Svedberg equation.

The reduced viscosity was related to the concentration according to the equation

$$\eta_{sp}/c = [\eta] + 0.305\ [\eta]^2\ C \pm 0.21,$$

where the intrinsic viscosity, $[\eta]$, was 15.1. The calculated value for the Scheraga-Mandelkern constant was 2.04×10^6. These data are compared to those for k-casein in other solvents in Table 9.

Physical Properties of Reduced k-Casein

Sedimentation Characteristics in 5.0 M GU

The experiments were performed with the PMB derivative of reduced k-casein to prevent re-oxidation of the disulfide bonds. Apparent molecular weights for the derivative remaining after removing aliquots for -SH group determination are shown as a function of the concentration in Figure 19 (filled circles). These values were obtained from the slopes of Van Holde-Bladwin plots which were linear for these equilibrium measurements. The apparent molecular weights, compared to those of k-casein in Table 8, did not show marked concentration

dependence. A molecular weight of 28,700 was obtained by least-squares extrapolation to zero concentration. A weight-average molecular weight of 21,000 was obtained for a reduced, purified Fraction S in 5.0 M GU at pH 8.4 and containing ME.

The diffusion and sedimentation coefficients were 5.8 Ficks and 1.3 S at a protein concentration of 9 mg/ml. A molecular weight of 20,000 was obtained when these values were inserted into the Svedberg equation. The concentration dependence of a later preparation of the PMB-derivative gave the result

$$S_{20,w} = 1.88\ (1 - 0.0106\ c).$$

However, equilibrium molecular weight analysis showed M_w = 34,000, M_z = 50,000, and a non-linear Van Holde-Baldwin plot, indicating either limited re-oxidation had occurred before the PMB was added or complete reduction was not achieved. This material was not used for -SH group analysis.

Sedimentation Characteristics in 67% Acetic Acid Adjusted to 0.15 M NaCl

In these studies, the protein was reduced in 5 M GU at pH 8.4. The solution was adjusted to pH 3 with glacial acetic acid and dialyzed against the solvent. There should be no re-oxidation of -SH groups under these conditions. The protein concentration was determined by measuring the area under the curve formed in the synthetic boundary cell. Concentration dependence of the apparent molecular weights is shown in Figure 19 (open circles). As observed for the case of the PMB-derivative in 5 M GU, the concentration dependence was not large. These values represent both the weight-average molecular weight for the entire cell contents and that obtained

from the slopes of the Van Holde-Baldwin plots. Since these plots were linear, the values obtained agreed within experimental error. Also the ratio M_z/M_w was approximately one as would be expected from the appearance of the Van Holde-Baldwin plots. The molecular weight obtained by least-squares extrapolation to zero concentration was 27,300. These results are compared to those for k-casein in the same solvent in Table 10.

The sedimentation-velocity data for reduced k-casein are represented by the relation

$$S_{20,w} = 1.26 \ (1 - 0.0146 \ c)$$

where $S_{20,w}$ is in Svedbergs and c in mg/ml. The diffusion coefficient, obtained at 6.4 mg/ml protein concentration, was 5.7 Ficks. Assuming the $D_{20,w}$ is not concentration dependent, a molecular weight ($M_{s,D}$) of 20,000 was calculated.

Determination of Sulfhydryl Groups

The nitroprusside test for free sulfhydryl groups performed in 5 M GU gave negative results for purified Preparation No. 3A and purified Fraction S. following treatment with ME, the test was positive and the PMB titration experiments showed the presence of 2-3 -SH groups per 28,000 g of k-casein. Analysis of total sulfur and methionine showed that by difference, 3 cysteine/28,000 existed. k-Casein held at pH 12 for 24-48 hours at 20° C did not show any SH groups upon subsequent reduction with ME and titration with PMB. The results are summarized in Table 11.

TABLE 4

The amino acid composition of k-caseins

Amino acid	g/100 g[a]	W_i[b]	Residues/28,000 g		
			This study[c]	Jolles 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	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

TABLE 4 - Continued

[a] Based on a nitrogen content of 15.3%.

[b] Weight per cent of the i^{th} amino acid residue.

[c] Analysis provided through the courtesy of Drs. J. P. Riehm, J. C. 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 NANA and phosphorus (based on the H_2PO_3 residue) as well as the values for Cys, Try, galactose, and galactosamine reported by Jollès *et al.* (1962).

TABLE 5

Molecular weights of k-casein from chemical analysis

Component	Percentage	Minimum molecular weight	Number of residues	Molecular weight
Sialic acid (NANA)	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).

TABLE 6

Sedimentation coefficients of k-caseins at neutral pH

Concentration (mg/ml)	S_{20} X 10^{13}	Temperature °C	Solvent
6.0[a]	14.5	3	phosphate buffer, 0.1 $\Gamma/2$
6.4[a]	13.9	25	phosphate buffer, 0.2 $\Gamma/2$
8.1[a]	13.02	25	0.5 M NaCl
8.2[a]	13.41	25	phosphate buffer, 0.2 $\Gamma/2$
10.0[b]	13.44	24	phosphate buffer, 0.1 $\Gamma/2$
10.0[a]	13.11	25	phosphate buffer, 0.2 $\Gamma/2$

[a] Purified preparation No. 3A.

[b] Preparation obtained by the method of McKenzie and Wake.

TABLE 7

Sedimentation coefficients of purified Preparation No. 3A in buffers containing SDS

Buffer	SDS (mg/ml)	Protein (mg/ml)	SDS (g/g protein)	$S_{20,w} \times 10^{13}$
Veronal	2.5	8	0.31	2.28[a]
pH 8.6	5.0	8	0.63	2.11[a]
$\Gamma/2 = 0.1$	10.0	5	2.00	2.28, 1.14[b]
	10.0	8	1.25	2.19[c]
	10.0	10	1.00	2.35[c]
	20.0	9	2.22	2.32[a], 0.84[b]
Tris	0	8.2	--	8.29[a]
pH 9.25	10	8.2	1.22	2.00[c]
$\Gamma/2 = 0.1$	20	8.3	2.41	2.26, 0.98[b]
	30	8.3	3.61	1.10, 0.80[b]
	30	8.1	3.70	1.2[a,d]
	40	6.0	6.67	1.1[e]

[a] Not corrected to water.

[b] Value for boundary due to SDS micelles.

[c] Boundary due to SDS micelles was observed.

[d] Free SDS removed by chilling to 0° C and centrifuging out the precipitated SDS.

[e] Performed in synthetic boundary cell by layering, on the solution, buffer containing 40 mg/ml SDS which was dialyzed against the solution.

TABLE 8

Equilibrium molecular weight data for k- and reduced k-casein in 5.0 M GU, pH 4.8

k-Casein			Reduced k-Casein	
Concentration (mg/ml)	$M_w \times 10^{-4}$	M_z/M_w	Concentration (mg/ml)	$M_w \times 10^{-4}$
0	12.61		0	2.87
3.0	11.28[a]	1.94[a]	3	2.77[d]
5.5	9.44	1.18	6	2.52[d]
7.2	8.73[a]	1.18[a]	9	2.49[d]
10.3	7.38[a]	1.17[a]	7[b]	2.1[b,c]

[a] Average of several determinations.

[b] Fraction S (Waugh and von Hippel, 1956), purified by ultracentrifugation and elution from Sephadex G-75.

[c] Solvent contained 7 µl/ml ME at pH 8.4.

[d] PMB derivative.

TABLE 9

Hydrodynamic properties of k-casein in 5.0 M GU, 67% acetic acid - 0.15 M NaCl and pH 12.2 phosphate buffer

Property	5.0 M GU pH 4.8	67% Acetic Acid	Phosphate Buffer (pH 12.2, 0.19 Γ/2)
$S^{\circ}_{20,w} \times 10^{13}$	3.18	2.7	1.4
k[a]	0.0178	0.0302	0.0172
$D_{20,w} \times 10^{7}$	3.1 (10.3 mg/ml)	2.4 (c = 0)	5.8 (8 mg/ml)
$[\eta]$ (ml/g)	31	35	15.1
Huggins constant	0.82	0.50	0.305
f/fo[b]	2.7	3.2	2.3
$\beta \times 10^{-6}$	2.00[c]	1.8[c]	2.04[d]
$M_{s,D}$	75,000 (10.3 mg/ml)	101,000 (c = 0)	22,300 (c = 0)

[a] From the equation $S_{20,w} = S^{\circ}_{20,w} (1 - kC)$

[b] Calculated from sedimentation-velocity and molecular weight data.

[c] A molecular weight of 120,000 was used in the calculation.

[d] A molecular weight of 24,000 was used in the calculation.

TABLE 10

Equilibrium molecular weight data for k- and reduced k-casein in 67% acetic acid - 0.15 M NaCl

k-Casein			Reduced k-Casein		
Concentration (mg/ml)	M_w X 10^{-4}	M_z/M_w	Concentration (mg/ml)	M_w X 10^{-4}	M_z/M_w
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[b]	8.71[a,b]	1.14[a,b]			
7.34	8.75	0.98			
11.00	7.03	0.91			

[a] Value for one calculation.

[b] Fraction S (Waugh and von Hippel, 1956), purified by ultracentrifugation and elution from G-75 Sephadex.

TABLE 11

Determination of the total sulfhydryl groups

Protein	Method of SH Group Analysis	Number SH/ 28,000 g	Nitroprusside
Reduced k-casein	Titration of unreacted PMB[a] with GSH[b]		+
	Trial 1 (18.9 mg)[c]	1.93	
	Trail 2 (15.5 mg)[c]	2.67	
	Direct determination of Mercaptide at pH 9.5	1.82	
k-Casein	Sulfur and methionine analysis	2.98	-
k-Casein prepared by the method of Waugh and von Hippel (1956)			-
k-Casein treated at pH 12 followed by reduction	Titration of unreacted PMB with GSH	0.1	-

[a] p-Mercuribenzoate

[b] Glutathione

[c] Total amount of protein reacted with excess PMB

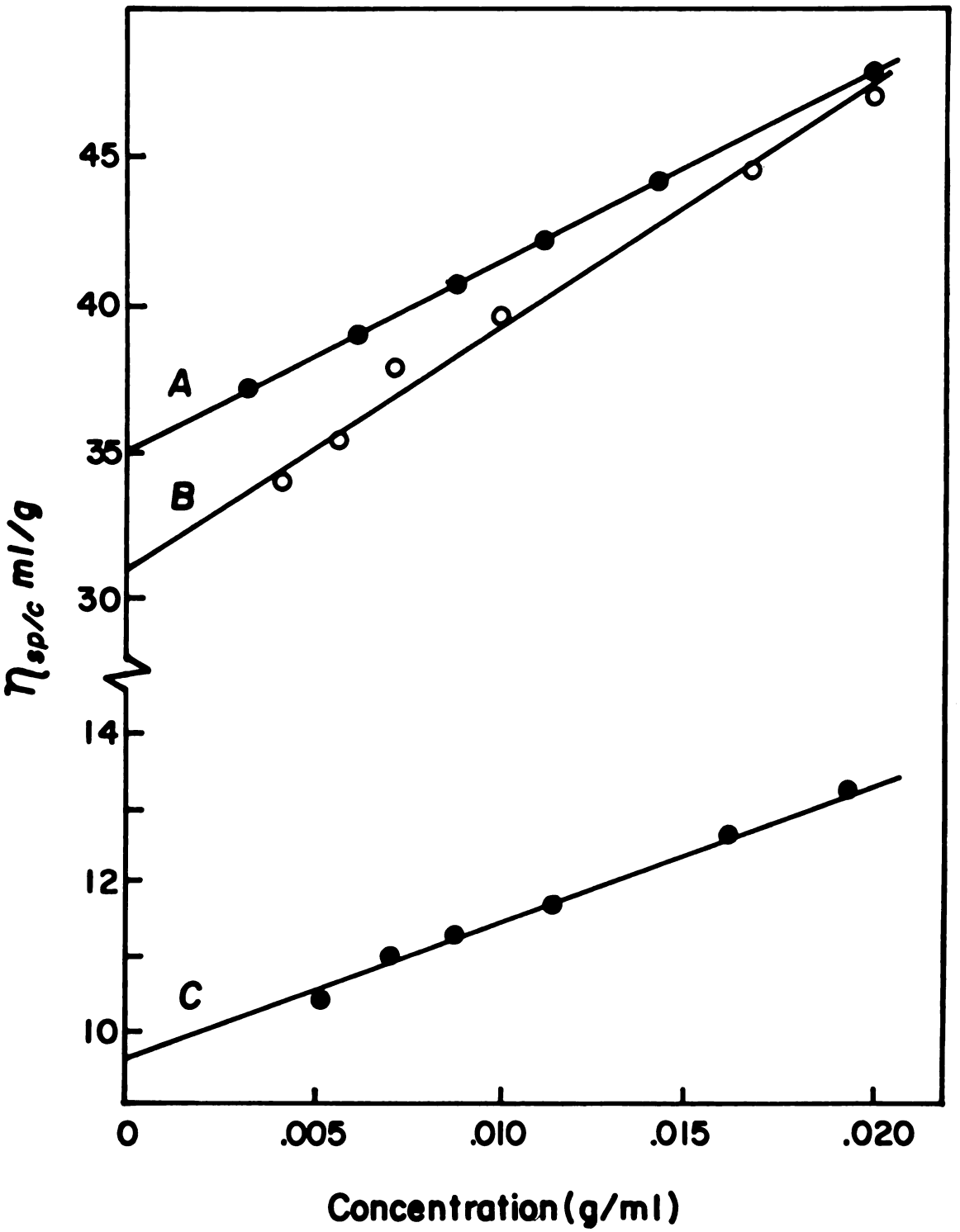


Figure 12. A plot of reduced viscosity (ml/g) for k-casein in A) 67% acetic acid - 0.15 M NaCl, B) 5.0 M guanidine·HCl, and C) 0.1 M NaCl.

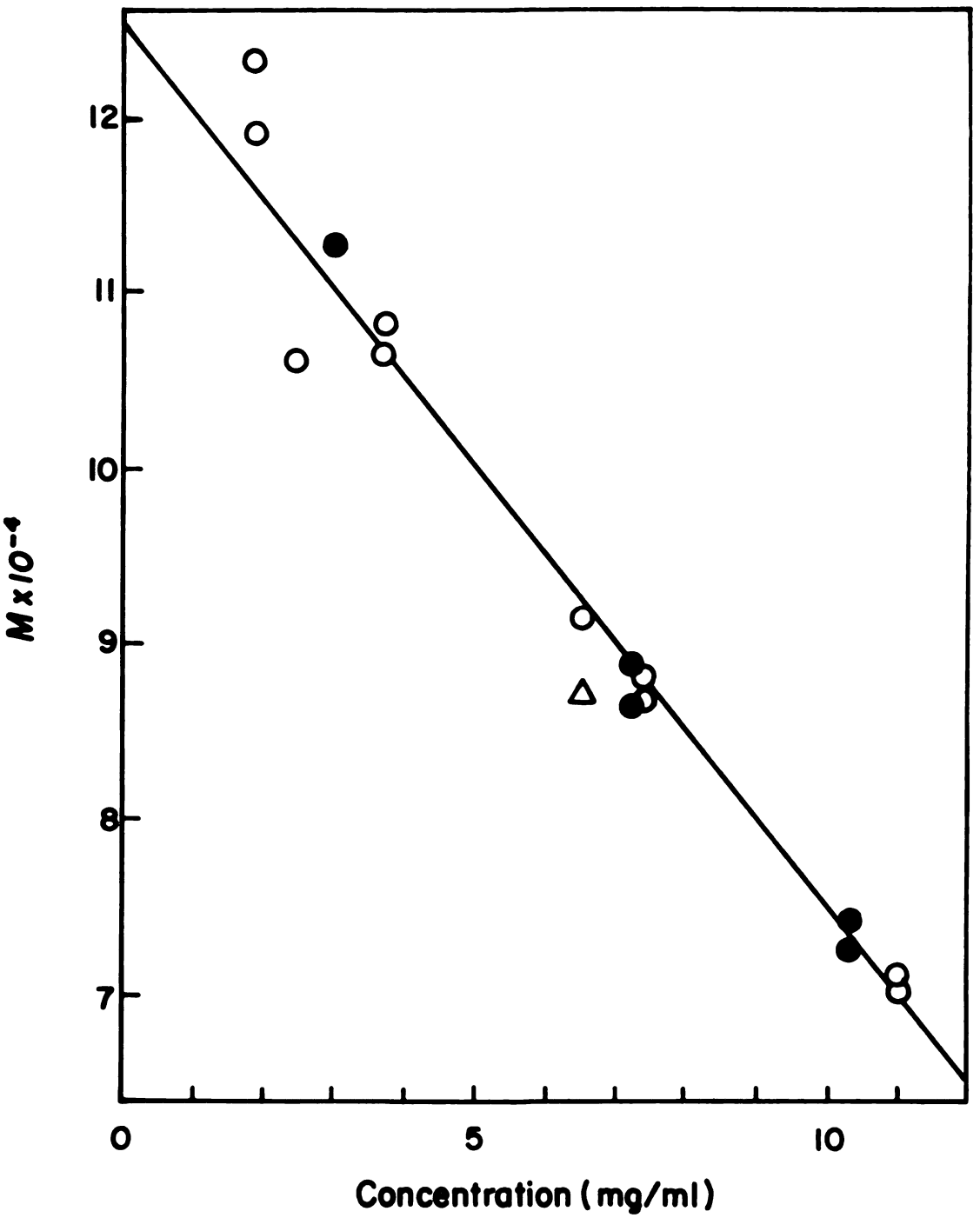


Figure 13. Plot showing the concentration dependence of the apparent molecular weight of k-casein in 5.0 M guanidine.HCl and 67% acetic acid - 0.15 M NaCl. Legend: O, M_w in 67% acetic acid; ●, M_w in 5.0 M guanidine·HCl; △, M_w in 67% acetic acid for purified Fraction S (Waugh and von Hippel, 1956).

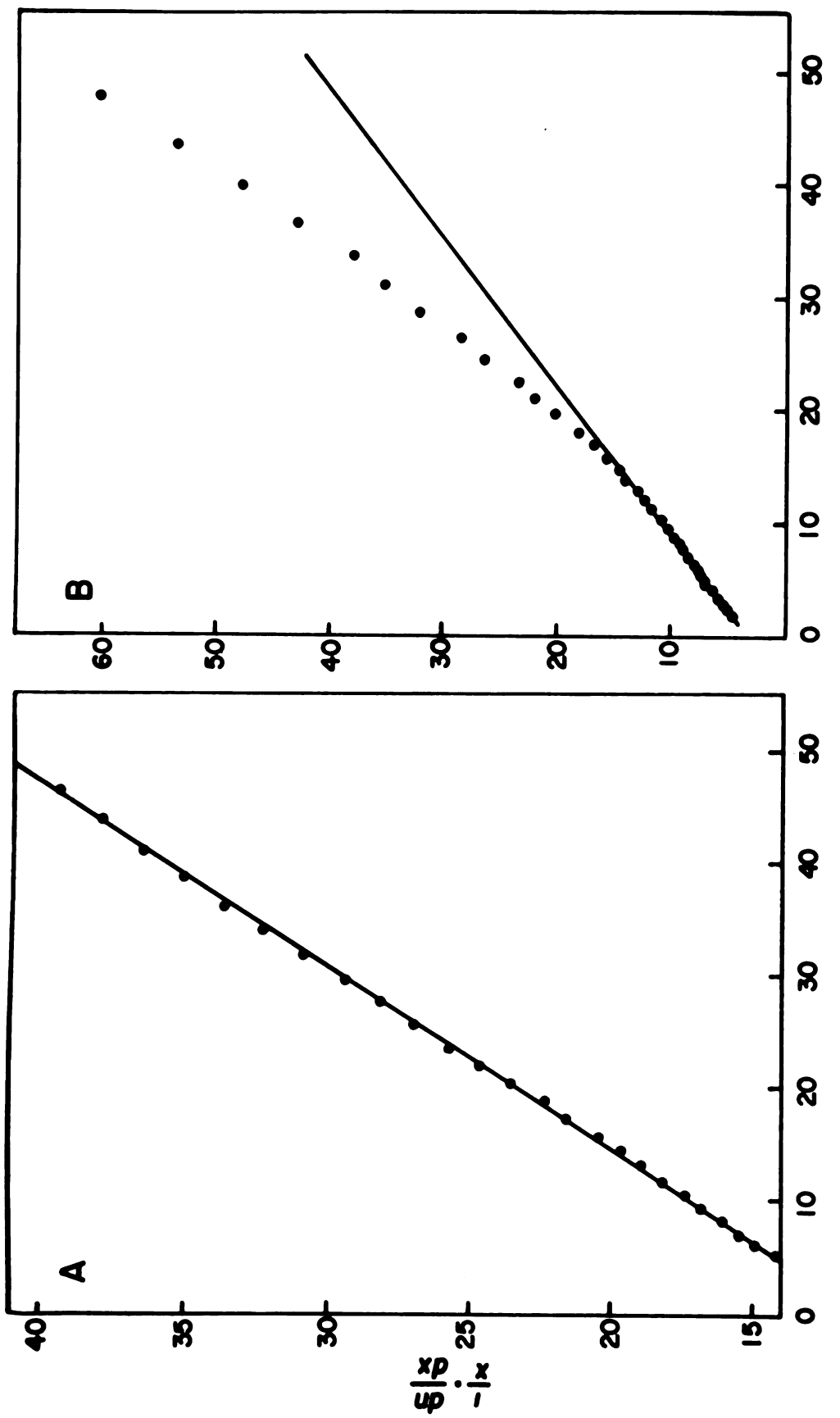


Figure 14. Van Holde-Baldwin plots of short-column equilibrium data for k-casein. A) 7.34 mg/ml in 67% acetic acid - 0.15 M NaCl. B) 3.0 mg/ml in 5.0 M guanidine·HCl.

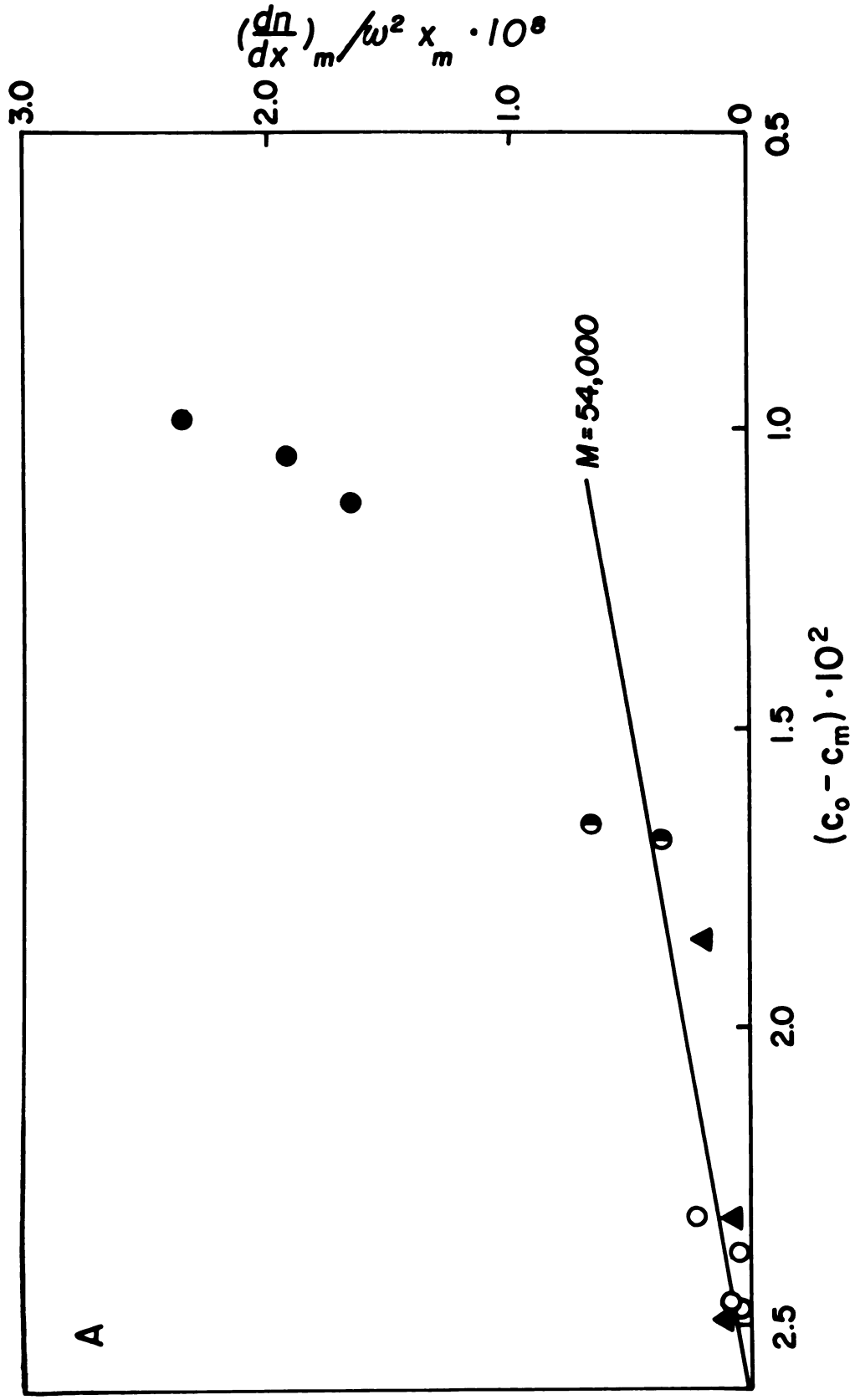


Figure 15. Trautman plot of approach-to-equilibrium data for k-casein in 5.0M guanidine·HCl. Legend: ○, 42,040 RPM; ▲, 33,450 RPM; ●, 20,410 RPM; ■, 12,590 RPM.

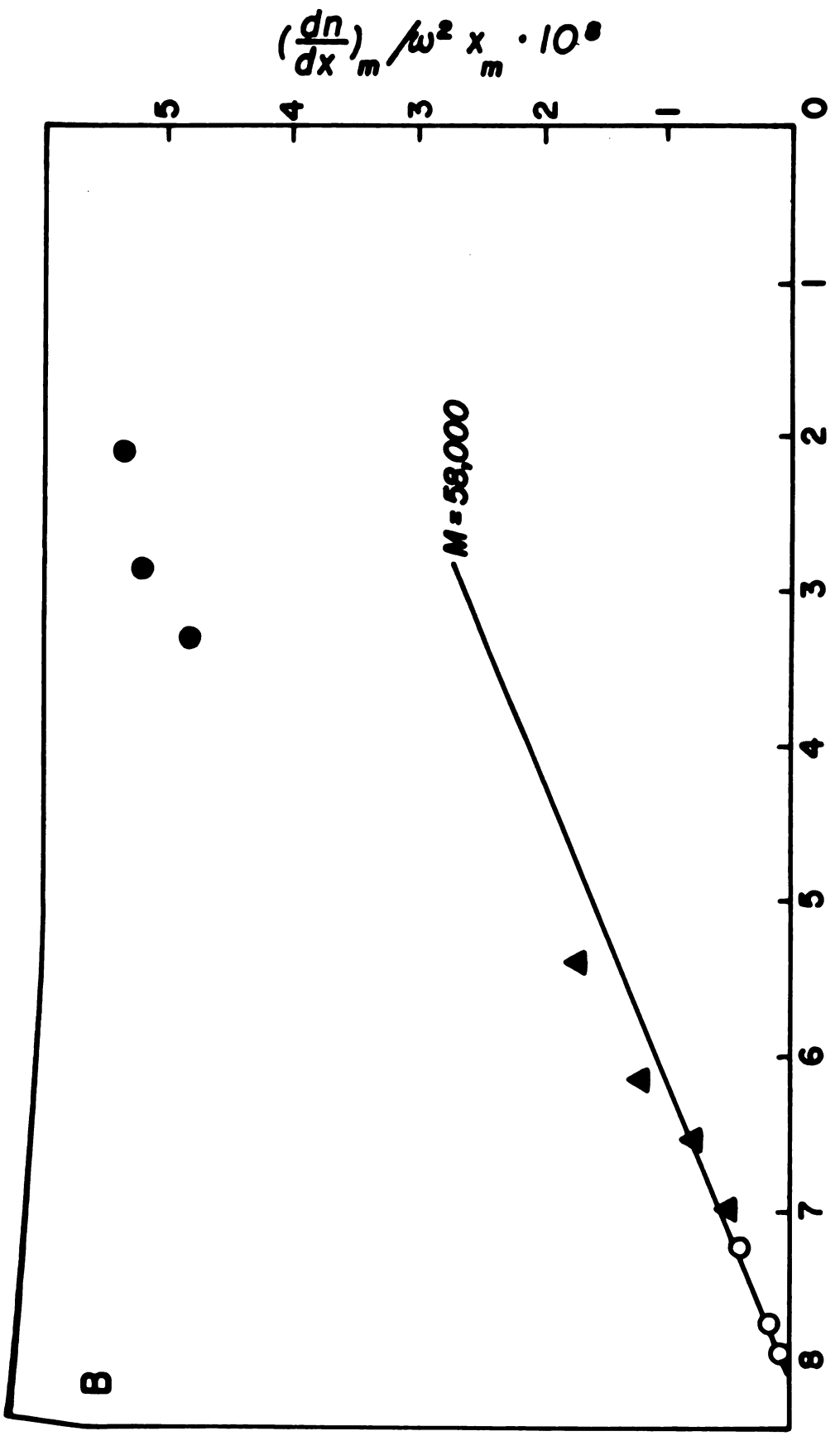


Figure 16. Trautman plot of approach-to-equilibrium data for k-casein in 33% acetic acid - 0.15 M NaCl. Legend: ○, 42,040 RPM; ▲, 29,500 RPM; ●, 14,290 RPM.

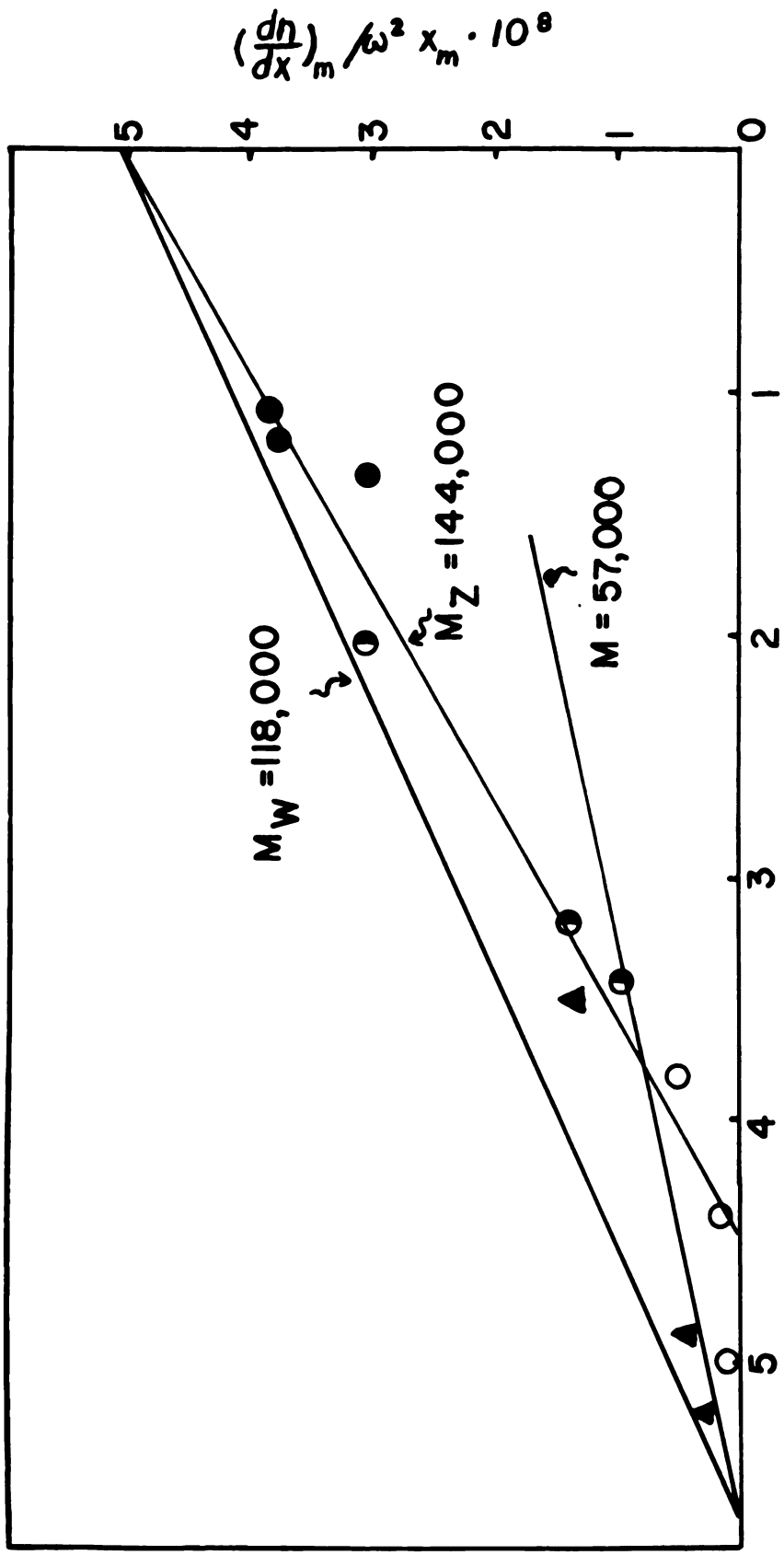


Figure 17. Trautman plot of appraoch-to-equilibrium data for k-casein in 7.0 M urea, pH 8.5, 25° C. Legend: ○, 42,040 RPM; ▲, 29,500 RPM; ●, 20,410 RPM; ●, 12,590 RPM.

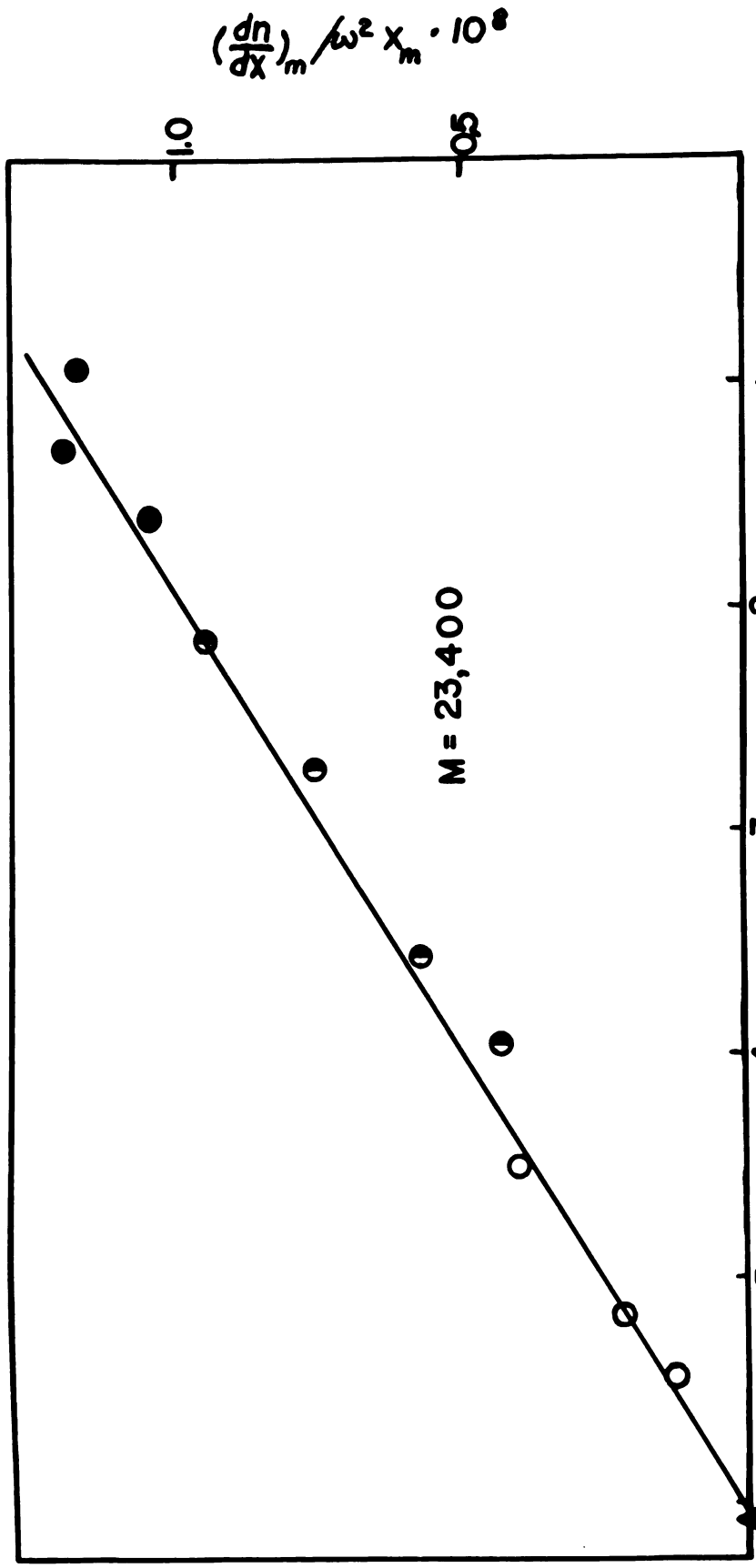


Figure 18. Trautman plot of approach-to-equilibrium data for k-casein in pH 12.2 phosphate buffer; 25° C. Legend: ▲, initial concentration; ○, 42,040 RPM; ●, 29,500 RPM; ●, 16,200 RPM.

Figure 19. Plot showing the concentration dependence of the apparent molecular weights in dissociating solvents for reduced k-casein and k-casein exposed to pH 12. Legend: 0, M_w for reduced k-casein in 67% acetic acid - 0.15 M NaCl; ●, M_w for PMB-k-casein in 5.0 M guanidine·HCl; Δ, M_w for purified Fraction S (Waugh and von Hippel, 1956) in 5.0 M guanidine, pH 8.4, containing 7 μl/ml 2-mercaptoethanol; ◒ M from Trautman plot of approach-to-equilibrium data for k-casein in pH 12.2 phosphate buffer, $\Gamma/2 = 0.19$; ◐, M_w for k-casein treated at pH 12.2, then dialyzed against 5.0 M guanidine·HCl.

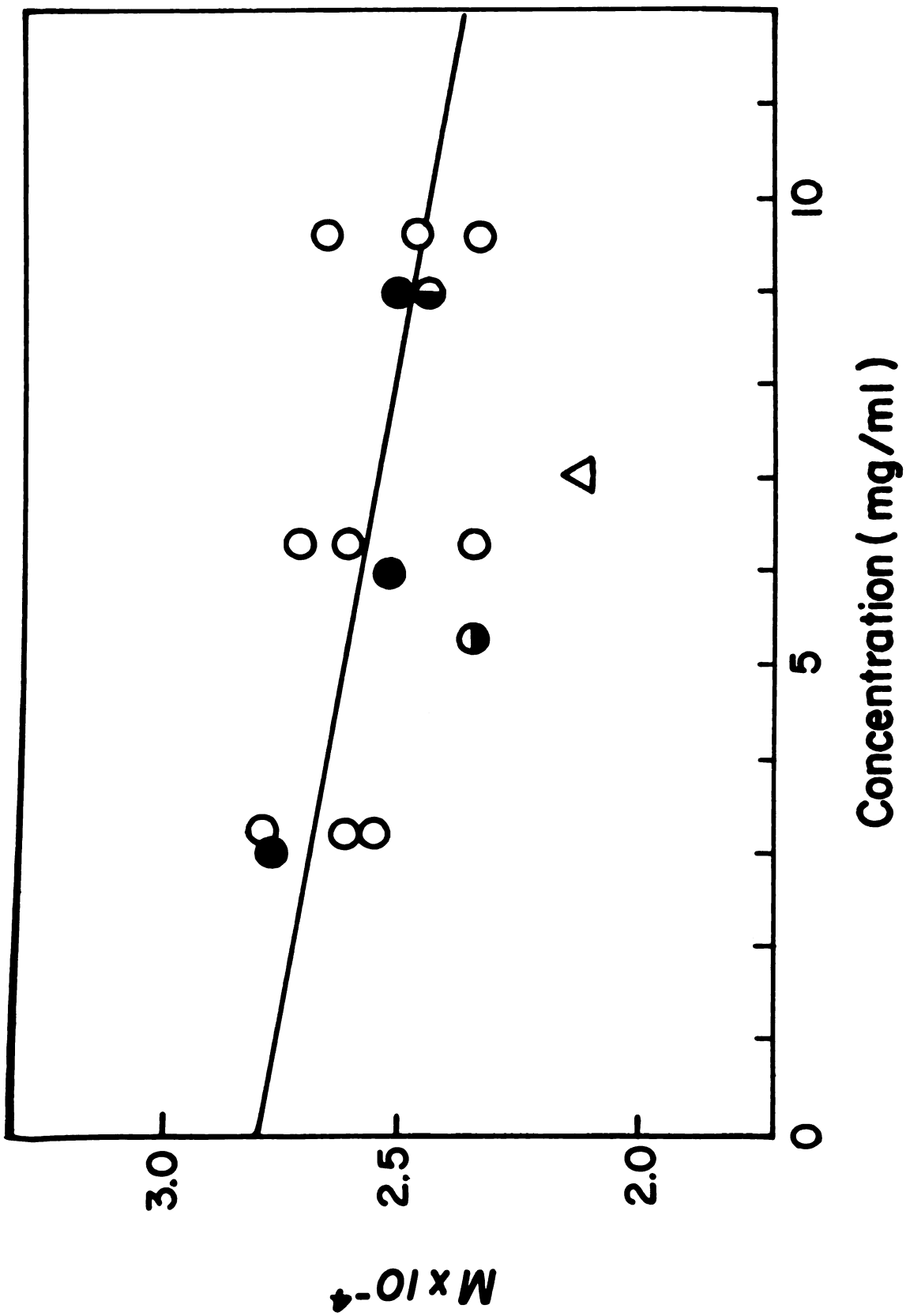


Figure 19

DISCUSSION

Composition of k-Casein

A comparison of the amino acid composition of purified Preparation No. 3A with that of Jollès et al. (1962) for a McKenzie-Wake preparation showed good agreement. The analyses, including the carbohydrate and phosphorus content, accounted for 95% of the total protein. Jollès et al. (1962) accounted for only 85% of the residues in their analysis of k-casein. Perhaps a more thorough analysis of the carbohydrate moiety would account for the remaining portion. The total number of cationic and anionic groups per 28,000 g was estimated to be 22 and 58, respectively, assuming that a) the phosphoamino acid side chains were dibasic anionic groups and b) ignoring the amide nitrogen. When the 32 amide nitrogens are considered, then, there exists but four excess anionic groups. The large amount of proline is particularly noteworthy.

The 1.4% of NANA found for purified Preparation No. 3A lies in the range reported for other k-casein preparations. Marier et al. (1963) reported values of 0.65% and 1.25% for a McKenzie-Wake preparation, whereas, Thompson and Pepper (1962) and Jollès et al. (1962) reported values of 2.5% and 2.4%, respectively, for preparations by the same method. A modification of the McKenzie-Wake preparation yielded k-casein containing 1.8% NANA (Nitschmann and Beeby, 1963). Also, Marier, et al. (1963) gave a value of 2.14% for a preparation obtained by the Swaisgood-Brunner method as previously published but not purified as described herein. Urea-starch gel electrophoresis showed more of the leading bands in their preparation. The extent of

variation due to analytical procedures and that due to differences in the preparations can only be speculated. Possibly some of the contaminating proteins found in k-casein preparations contain NANA. This postulate is supported by the observation of Malpress (1961) that only 68% of the NANA in whole casein was released by rennin whereas Marier et al. (1963) found approximately 90% of the NANA was released from a Swaisgood-Brunner preparation of k-casein.

To some extent the phosphorus content appears to be related to the purity of k-casein preparations. Waugh's Fraction S contained about 0.5% P. The crude k-casein prepared by Long (1958) contained 0.46% P, but following five centrifugations it contained 0.33% P. Purer preparations had a phosphorus content of approximately 0.22% (Jollès et al., 1962; Thompson and Pepper, 1962; Waugh, 1958). Preparations obtained during this study varied from approximately 0.35% P for crude k-caseins to 0.22% P for the purified material.

The composition of α_3-casein is similar to that of k-casein. This protein fraction is undoubtedly rich in k-casein as evidenced by its amino acid composition, phosphorus and NANA contents, reaction with rennin, and ability to stabilize α_s-casein. Nevertheless, there are some noteworthy differences between these two proteins: a) the phosphorus content of α_3-casein (0.35%) is similar to that of crude k-casein although electrophoresis and sedimentation-velocity experiments showed only one boundary, b) its sedimentation coefficient (23 S) is much larger than that for k-casein, c) it is much less soluble at pH 6.9 (0.26%), and d) its threonine content is significantly lower. Interestingly, this protein was prepared by the same procedure employed by Long (1958) with the following exceptions; a) only one centrifugation

at low speed was used to remove the α_s-casein and b) the final ultracentrifugations were performed in the absence of salt. Possibly the interaction observed by Long (1958) in the presence of salt participates in the formation of a stable complex in the absence of salt. The fact that repeated ultracentrifugation in salt solutions does not concentrate k-casein preparations beyond a certain degree of purity was observed by Hipp et al. (1961) and, also, during the course of this study. The formation of stable complexes between k-casein and one or more of the λ-casein constituents would explain the discrepancies listed above. For example, the greatest difference between the amino acid composition of the λ-casein fraction and k-casein as reported in this thesis was the lower content of threonine in λ-casein. Furthermore, Hipp et al. (1961) observed that the exposure of α_s-casein to pH 11.8 resulted in the formation of a number of electrophoretic peaks at pH 10.1 where previously only one peak was observed. In view of the above argument, this observation would indicate that the associated complex of casein's was dissociated in the high pH environment (Waugh and von Hippel, 1956).

Molecular Size and Interactions of k-Casein

Polymer Size of k-Casein in Neutral Salt Solutions

k-Casein forms polymers in neutral buffers or NaCl solutions which are relatively independent of temperature or ionic strength. These polymers are not entirely uniform with respect to size as evidenced by the skewness of the sedimenting boundary. Nevertheless, the interaction is unusually specific and an estimate of the aggregate size is noteworthy.

Although a direct determination of the molecular weight was not performed, an approximation can be obtained from the Scheraga-Mandelkern equation (Appendix II) since β is relatively insensitive to particle shape. Thus, using the equation given by Schachman (1959), a molecular weight of approximately 650,000 was calculated from the sedimentation coefficient and intrinsic viscosity. Therefore, the polymer seems to be composed of 10 to 15 basic units (M = 56,000). One must not conclude that this aggregate is indigenous to the native micelle, however, this ability to form aggregates certainly demonstrates the proteins unusual capability for specific interaction.

Dissociation of the k-Casein Polymers and the Properties of the Units Obtained

Sedimentation, diffusion, and viscosity characteristics of k-casein in dissociating solvents. The polymer size of k-casein was significantly reduced when dispersed in solvents containing SDS, 7.0 M urea, 5.0 M GU, concentrated acetic acid, or high concentrations of hydroxyl ion. Thus, the equilibrium molecular weights extrapolated to zero concentration in 67% acetic acid and 5.0 M GU were approximately 125,000. Although there was some indication of an increase in the apparent molecular weights at low concentrations (Figure 13), the protein was essentially monodispersed in 67% acetic acid. At low concentrations in 5.0 M GU and in 7.0 M urea or 33% acetic acid the protein exhibited properties characteristic of polydispersity. In 5.0 M GU and 7.0 M urea, the M_w was similar to that obtained in 67% acetic acid but the M_z was nearly doubled. Determinations of the molecular weight of the light component in three solvents, i.e., 33% acetic acid, 5.0 M GU, and 7.0 M urea, resulted in an average value

of 56,000. These data may be interpreted to suggest that k-casein existed in these solvents in the form of both lower and higher polymer species. For example, lowering the concentration of acetic acid or lowering the concentration of protein in 5.0 M GU may favor a monomer-polymer equilibrium rather than the dimer form observed in 67% acetic acid. The basic unit of k-casein appears to have a molecular weight of approximately 56,000.

The molecular weight of k-casein in solutions containing 10 mg/ml SDS was roughly 100,000. The polymer size was probably similar to that in acetic acid, GU, and urea solutions since this calculation assumed one gram of SDS bound per gram of protein; a figure most likely over-estimated. However, the molecular weight at pH 12 was approximately 24,000 or about one-half the value for the basic unit. This result compares well with the value reported by McKenzie and Wake (1959b).

Molecular weight values were not corrected for charge effects or specific protein-solvent interaction--except for an estimate in the case of SDS--because of a lack of satisfactory correction values. However, the charge effect should not be large since the solutions contained relatively high concentrations of salt (e.g., $0.15\ \Gamma/2$) (Svedberg and Petersen, 1940; Schachman, 1959; Tanford, 1961). Preferential interactions with SDS and concentrated acetic acid should not effect the molecular weight greatly since their partial specific volumes are nearly the reciprocal of the density of the solution (Schachman, 1960). Kielley and Harrington (1960) found that $< 5\%$ GU was protein-bound for two dissimilar proteins (i.e., ribonuclease and myosin) dispersed in 5.0 M GU. Further, these investigators obtained a molecular weight of $13,000 \pm 500$ for ribonuclease which agrees with

the known value.. The fact that similar molecular weights were obtained in all of the solvents employed in this study suggests that large charge effects or preferential interactions were not operative.

These studies demonstrated that the polymers were not completely dissociated in any of the solvents employed except the pH 12 buffer. Although a smaller unit was present, a tendency towards larger polymer size was more apparent in 7.0 M urea, 33% acetic acid, and 5.0 M GU at low protein concentrations than in 67% acetic acid. Conceivably an unusually strong interaction occurs between the basic units of k-casein since most proteins would be completely dissociated under these conditions.

The sedimentation coefficients, especially in 67% acetic acid, exhibited a considerable concentration dependence. Presumably this characteristic can be attributed to the non-ideality of the system as demonstrated by the large dependence of the molecular weights on concentration. However, the diffusion coefficient in 67% acetic acid showed only a small increase with decreasing concentration. The intrinsic viscosities in 67% acetic acid and 5.0 M GU were greatly increased over that in neutral salt solutions or pH 12 phosphate buffer. This observation was similar to that of Harrup and Woods (1961) who found that the intrinsic viscosities of bovine serum albumin, egg albumin, β-lactoglobulin, lysozyme, and insulin were much greater in anhydrous formic acid than in aqueous solutions.

The calculated frictional ratios as well as the large intrinsic viscosities suggest that the molecules were extended. However, a combination of thermodynamic and hydrodynamic data in the form of the Scheraga-Manderkern constant indicates that the molecule underwent

an isotropic swelling rather than an extension in the polypeptide chains. The calculated values for β were 1.8×10^6, 2.00×10^6, and 2.04×10^6 in 67% acetic acid, 5.0 M GU, and pH 12 phosphate buffer, respectively. The theoretical minimum value is 2.12×10^6, however, the values reported here are within the experimental error of this minimum. Scheraga and Mandelkern (1953) discuss the case of horse serum albumin in urea solutions where the frictional ratio indicated an axial ratio of approximately 20 : 1, whereas the calculated β's ranged from 1.98 to 2.05×10^6. They concluded that the protein molecules swelled in the presence of urea. Values lower than the theoretical minimum have been reported for other proteins (Schachman, 1959; Yang, 1961). Harrup and Woods (1961), on the basis of light scattering data, suggested that isotropic swelling occurred in bovine serum albumin rather than a linear extension of the chains in anhydrous formic acid.

The dissociation of k-casein in 67% acetic acid and 5.0 M GU was reversible with respect to the sedimentation-velocity characteristics. k-Casein which was held for two weeks in 67% acetic acid at room temperature exhibited the same NANA content and reaction with rennin following dialysis against water as it did prior to the exposure to the dissociating system. Holding the protein in 5.0 M GU or 7.0 M urea followed by dialysis against water yielded a k-casein solution which reacted normally with rennin. Some difficulty was experienced in completely removing the bound SDS from the protein as indicated by a lower sedimentation coefficient (e.g., $\simeq$ 10S).

Sedimentation and diffusion characteristics of reduced k-casein in dissociating solvents. Following cleavage of the disulfide bonds with ME, the molecular weight of k-casein was approximately 28,000 in

both 5.0 M GU and 67% acetic acid. The protein appeared monodispersed as evidenced by linear Van Holde-Baldwin plots and ratios of M_z/M_w approaching one. As in the case of k-casein, the molecular weights were not corrected for charge effects or preferential interactions. Concentration dependence was not marked for either the molecular weights or the sedimentation coefficients. Diffusion coefficients were determined at only one concentration in both solvents giving values of 5.8 Ficks at 9 mg/ml in 5.0 M GU and 5.7 Ficks at 6.4 mg/ml in 67% acetic acid.

Comparison of k-casein and reduced k-casein. Reduction of the disulfide bonds clearly caused a decrease in the observed molecular weight of k-casein. This fact was obvious even from a qualitative observation of the sedimentation-equilibrium patterns, see Figure 20. Furthermore, a comparison of the sedimentation and diffusion coefficients showed that cleavage of the disulfide bonds caused a reduction in the $S_{20,w}$ and an increase in the $D_{20,w}$. The concentration dependency of the sedimentation coefficients for reduced k-casein were approximately half that for k-casein. This was as expected since the apparent molecular weights for reduced k-casein were much less concentration dependent. These data are summarized in Table 12.

The basic unit (M = 56,000) of k-casein observed in various dissociating solvents appears to be composed of two sub-units having a molecular weight of approximately 28,000. The proposed sub-unit weight is in accord with the chemical analyses which indicated an average minimum molecular weight of 24,300. Furthermore, it is proposed that the two sub-units are joined by disulfide bonds. This postulate is supported by the presence of three -SH groups per 28,000 g

as determined by PMB titrations, analyses for total sulfur and methionine, and the cysteic acid values reported by Jolles et al. (1962) and Hipp et al. (1961). Since k-casein does not contain free -SH groups, an odd number of -SH groups per sub-unit weight precludes at least one inter-molecular disulfide bond. The results obtained were not a consequence of the particular method of preparation employed in this study since the same observations were made on a freshly prepared, purified Fraction S preparation.

A comparison of the properties of k-casein in pH 12 phosphate buffer to those of reduced k-casein indicated that the disulfide bonds were destroyed in this buffer. Also, k-casein dissolved in the phosphate buffer and then dialyzed against 5.0 M GU exhibited an equilibrium molecular weight of 24,500 at a protein concentration of 9 mg/ml, see Figure 19. Chemical evidence showing that alkaline-treated protein subsequently reduced with ME did not give the nitroprusside reaction or react with PMB substantiated the conclusions derived from the physical studies.

These results are not surprising considering the lability of disulfide bonds in proteins to alkali (Cecil and McPhee, 1959). Brown, Delaney, Levine and Van Vunakis (1959) observed an odor of hydrogen sulfide upon neutralization of aqueous solutions of ribonuclease which had been exposed to pH 12.7 for 30 min at room temperature. More recently, Young and Potts (1963) measured a 70% loss of half-cystine in ribonuclease after two hours at pH 11 in 5 M guanidine.

Further evidence in favor of a basic unit weight for k-casein of approximately 56,000 comes from the work of Garnier et al. (1962) and Beeby (1963). These researchers proposed that k-casein had a molecular

weight of 55,000 and 50,000, respectively. They based this conclusion on data pertaining to the release of protons and GMP resulting from the action of rennin on k-casein. If, as proposed by these workers, only one GMP is released per 56,000, then the two sub-units must be different but of approximately the same weight since the equilibrium patterns of reduced k-casein have shown it to be quite homogeneous. Further, a peptide other than GMP must be released by rennin since studies described earlier in this thesis showed that peptides of approximately 8,000 molecular weight were released from a 28,000 molecular weight unit.

Reduced k-casein did not show the same tendency to participate in a monomer-polymer equilibrium as did the non-reduced protein in the dissociating solvents employed. However, reduced k-casein in neutral phosphate buffer possessed the same sedimentation-velocity characteristics (S_{20} = 13 S) as the non-reduced. This result was expected since a typical sedimentation pattern was restored upon returning the protein to pH 7.0 from pH 12 (Waugh and von Hippel, 1956). Thus, the disulfide bonds may have some influence on the interactions of k-casein but certainly, the effect is not great.

Some other possible structures of k-casein. Recently Nitschmann and Beeby (1963) and Beeby (1963) proposed that k-casein was a complex and that the initial action of rennin was to break this complex. Seemingly, this proposal was based on the following observations. a) Fifteen per cent of their k-casein preparation was soluble at pH 4.7 and the precipitate became more insoluble at pH 7 with each precipitation. b) When a 6M urea solution of the protein was dialyzed

against pH 4.7 acetate buffer, a precipitate formed leaving 20% of the protein in the supernatant. The precipitate was partially insoluble at pH 7. c) The insoluble material at pH 7 appeared similar to para-k-casein and the soluble portion from a) and b) similar to GMP. d) And finally, very low rennin concentrations, the nitrogen and NANA initially appeared more rapidly in the pH 4.7 soluble portion than in the 12% TCA soluble material.

Despite these evidences of a k-casein complex, one must consider that neither the k-casein preparation nor the various fractions were well characterized. Furthermore, it is possible that λ-casein fraction was present in their preparations. Fraction S, prepared in this laboratory by a similar method, contained large quantities of the λ-casein fraction. A McKenzie-Wake preparation of k-casein also contained λ-casein. The percentage of nitrogen reported for one of their preparations (Beeby, 1963) was 14.6%, whereas, the purified k-casein studied here contained 15.3%. Moreover, purified Preparation No. 3A did not precipitate when solutions in 5.0 M GU at pH 4.8, 7.0 M urea, SDS, or acetic acid were dialyzed against water. The physical characteristics and reaction with rennin were normal for k-casein which had been treated in GU, urea, or acetic acid. Furthermore, a molecular weight of something less than 50,000 was not observed for k-casein in 7.0 M urea as would be expected if a complex existed and was broken as was suggested.

Several alternative interpretations of the data of Nitschmann and Beeby are considered.

1. The material which was soluble at pH 4.7 as described above in a) and b) may be enriched with the

λ-casein fraction. Possibly the λ-casein fraction contained the NANA not found in the GMP (see previous discussion). Also, some of the constituents of this fraction must have a relatively low molecular weight as evidenced by an M_w = 20,000 and M_z = 90,000 in 5.0 M GU. These characteristics could cause the λ-casein fraction, or some component thereof, to appear similar to GMP. The fraction of the precipitate obtained in a) and b) above which was insoluble at pH 7 (similar to para-k-casein) may have been an irreversible aggregate of k-casein and λ-casein fraction similar to α_3-casein.

Interactions of the λ-casein fraction with k-casein would also explain the more rapid increase in the amount of nitrogen and NANA soluble at pH 4.7 when compared to that in 12% TCA following the action of rennin. Observations in this laboratory indicated that some k-casein may be soluble at pH 4.7, e.g., k-casein preparations containing 35-50% - or possibly less - of the λ-casein fraction does not cloud when treated with rennin in dilute salt solutions (< 0.1 M). The amount of NPN liberated definitely indicated that GMP was being released (Note: Nitschmann and Beeby stated that k-casein was not in the pH 4.7 supernatant since the solution did not cloud with rennin). Further, the supernatant obtained at pH 4.4 during the urea-TCA preparative method contained in addition to large proportions of β-casein a fraction similar to crude k-casein as evidenced by free-

boundary electrophoresis.

Thus, it can be argued that the NANA-containing material which is soluble in 12% TCA prior to the action of rennin may be one or more of the λ-casein constituents. This fraction may not be entirely soluble in 12% TCA under all conditions which would explain the conclusion arrived at by Wake (1959), namely that all of the GMP was not soluble. The initial stage of the action of rennin on k-casein would cleave the GMP from the protein, thus, increasing the ratio of λ-casein to unaltered k-casein. At pH 4.7, then, more of the k-casein in addition to λ-casein would remain soluble. In fact, Beeby (1963) found that the NANA/N ratio increased with time and suggested that "the intact sialic acid-containing component carries with it part of the insoluble components of the k-casein complex". However, only the initial proportion of the λ-casein fraction and the released GMP would be soluble in 12% TCA. After the complete reaction has occurred, the only NANA-containing proteins would be GMP and the λ-casein fraction. If any of the latter was not previously soluble in TCA, interaction with GMP could lead to complete solubility, thus, all of the NANA would be determined as soluble NANA.

2. The faster increase of pH 4.7 - soluble nitrogen and - NANA compared to that which was soluble in 12% TCA can be explained in another manner. The complete rennin action may be a result of two cleavages. Thus, the initial

cleavage would yield a large peptide of 12,000-16,000 molecular weight which is soluble at pH 4.7 but not in 12% TCA. An extended reaction would cleave this peptide approximately in half giving the GMP and another peptide. This would account for one proton, as suggested by Garnier et al. (1962) and a single GMP per 56,000 molecular weight unit. An assay of the total soluble peptides released indicated that units of 6,000-8,000 molecular weight were released per 28,000 molecular weight.

Nevertheless, if the complex exists as proposed by Nitschmann and Beeby (1963), then another interpretation of the physical data presented here is tenable, namely; that the complex should consist of two proteins of approximately the same molecular weight (28,000) since equilibrium studies of the reduced material indicated homogeneity. Secondly, the disulfide bonds probably would not be inter-molecular, but rather intra-molecularily located and possibly in only one of the proteins. And finally, the disulfide bonds must facilitate the complex formation since cleavage of these bonds resulted in reduction of the molecular weight to 28,000 units.

TABLE 12

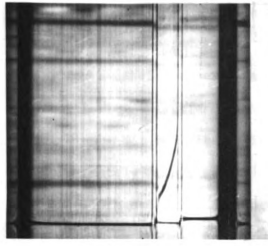
Summary of the physical properties of k-casein and reduced k-casein

Property	k-Casein				Reduced k-Casein		
	Phosphate Buffer pH 7.0	67% Acetic Acid-0.15 M NaCl	5.0 M GU	7.0 M Urea	Phosphate Buffer pH 12	67% Acetic Acid-0.15 M NaCl	5.0 M GU
M	650,000	124,000 58,000[b,c]	126,000 54,000[b]	118,000[a] 57,000[b]	24,000	27,300	28,700
$S^{\circ}_{20,w} \times 10^{13}$	15.6	2.7	3.18	---	1.4	1.26	1.88
k	0.0165	0.0302	0.0178	---	0.0172	0.0146	0.0106
$D_{20,w} \times 10^{7}$	---	2.3	3.1	---	5.8	5.7	5.8
$[\eta]$ (ml/g)	9.5	35	31	---	15.1	---	---
f/f_o	---	3.2	2.7	---	2.3	2.6	1.8
$\beta \times 10^{-6}$	---	1.8	2.0	---	2.04	---	---

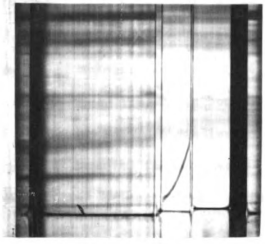
[a] Weight-average molecular weight for a protein concentration of 5 mg/ml.
[b] Molecular weight of the light component as determined from a Trautman plot.
[c] Solvent used for this determination was 33% acetic acid -0.15 M NaCl.

Figure 20. Sedimentation-equilibrium patterns for k-casein and reduced k-casein in 67% acetic acid - 0.15 M NaCl and 5.0 M guanidine.HCl. All experiments were performed at 25.0° C and a schlieren diaphragm angle of 70°. The protein concentrations were: top left, 7.34 mg/ml; top right, 7.2 mg/ml; bottom left, 6.3 mg/ml; and bottom right, 6 mg/ml.

KAPPA-CASEIN

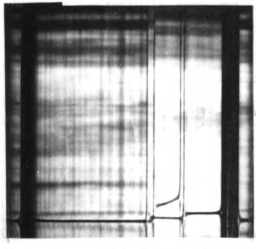


12,590 RPM
67% ACETIC ACID
0.15 M NaCl

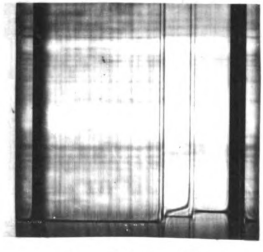


12,590 RPM
5 M GUANIDINE·HCl

REDUCED KAPPA-CASEIN



14,290 RPM
67% ACETIC ACID
0.15 M NaCl



16,200 RPM
5 M GUANIDINE·HCl

Figure 20

SUMMARY OF PART II

The amino acid composition of the preparation of k-casein described in this thesis compared favorably with that reported by Jollès *et al.* (1962) for k-casein prepared by the method of McKenzie and Wake (1961). The equilibrium molecular weight was approximately 125,000 in both 5.0 M guanidine • HCl and 67% acetic acid — 0.15 M NaCl. A smaller unit of approximately 56,000 molecular weight was detected in 33% acetic acid — 0.15 M NaCl, 7.0 M urea, and at low protein concentrations in 5.0 M guanidine • HCl. Reduction of the disulfide bonds of k-casein caused a decrease in the molecular weight to 28,000 in both 5.0 M guanidine • HCl and 67% acetic acid — 0.15 M NaCl. Approximately 3 — SH groups per 28,000 g of protein were indicated by a) total sulfur and methionine analyses, b) titration of the reduced k-casein, with p-mercuribenzoate, and c) the cysteic acid content as reported by Jollès *et al.* (1962).

These data are consistent with the interpretation that the basic unit of k-casein is composed of two polypeptide chains having a molecular weight of 28,000 and joined by at least one disulfide bond.

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APPENDIX I

Composition of the Buffers

The folowing quantities were made to one liter with redistilled water:

1. Hydrochloric acid - sodium chloride; pH 2.3; $\Gamma/2 = 0.1$.

   5.85 g NaCl

   Adjusted pH with 0.1 N HCl

2. Acetic acid - sodium acetate; $\Gamma/2 = 0.1$

   2.72 g sodium acetate

   Adjusted the solution to the desired pH with dilute acetic acid, ie. pH 4.6, 4.7, 5.1, 5.3, and 5.5.

3. Sodium phosphate; pH 6.0; $\Gamma/2 = 0.1$

   2.92 g NaCl

   4.68 g $NaH_2PO_4 \cdot H_2O$

   0.763 g Na_2HPO_4

4. Sodium phosphate; pH 6.5; $\Gamma/2 = 0.1$

   2.92 g NaCl

   2.75 g $NaH_2PO_4 \cdot H_2O$

   1.42 g Na_2HPO_4

5. Sodium phosphate; pH 6.7; $\Gamma/2 = 0.1$

   2.92 g NaCl

   1.73 g $NaH_2PO_4 \cdot H_2O$

   1.78 g Na_2HPO_4

6. Sodium phosphate; pH 7.0; $\Gamma/2 = 0.1$

   2.92 g NaCl

   1.06 g $NaH_2PO_4 \cdot H_2O$

   2.02 g Na_2HPO_4

7. Potassium phosphate; pH 7.0; $\Gamma/2 = 0.2$

   3.95 g KCl

   3.63 g KH_2PO_4

   6.97 g K_2HPO_4

8. Sodium phosphate; pH 7.5; $\Gamma/2 = 0.1$

   2.92 g NaCl

   0.43 g $NaH_2PO_4 \cdot H_2O$

   2.22 g Na_2HPO_4

9. Sodium veronal - hydrochloric acid; pH 8.3; $\Gamma/2 = 0.1$

   20.6 g sodium veronal

   Adjusted pH with 2 N HCl

10. Veronal; pH 8.6; $\Gamma/2 = 0.1$

    20.6 g sodium veronal

    2.797 g veronal

11. Potassium phosphate; pH 12.3; $\Gamma/2 = 0.19$

    3.22 g KOH

    8.71 g K_2HPO_4

Properties of the Dissociating Solvents Used for Molecular Weight Calculations or Correction of the Sedimentation Coefficients to Water

The densities were determined in 25 ml pycnometers and the viscosities in a Cannon-Ubbelohde semi-micro dilution viscometer at 25.0 ± 0.01° C.

Appendix Table 1

Densities and relative viscosities of some of the dissociating solvents

Solvent	η/η_w	ρ
67% acetic acid - 0.15 M NaCl	2.574	1.0681
33% acetic acid - 0.15 M NaCl	--	1.0445
7.0 M urea	--	1.1034
5.0 M GU	1.437	1.1198
Veronal buffer, pH 8.6 - 10 mg/ml SDS	1.101	1.0065

APPENDIX II

Introduction

The methods used to evaluate the ultracentrifugal data are presented in this section. The theoretical development of the required equations is presented to provide a firm basis of understanding for their applications. The following material does not represent an original contribution of this writer but is presented for the purpose of describing the use of the theoretical equations in this study. For a more thorough and extensive treatment of the theory, the reader is referred to the following references: Fujita (1962), Schachman (1959), Tanford (1961), Williams, Van Holde, Baldwin, and Fujita (1958), Baldwin and Van Holde (1960), Van Holde and Baldwin (1958), Trautman (1956), Svedberg and Peterson (1940), and Wales (1961).

Notation

The symbols $\underline{r}$ and $\underline{x}$ have been commonly used for the distance from the center of rotation. Since the ultracentrifuge cell is sector-shaped, the equations are written in cylindrical coordinates, therefore the symbol $\underline{r}$ will be used in this section (In Part II, $\underline{x}$ was used in several of the Figures.). The following symbols appear in this Appendix:

a' - The optical path length of the cell.

b' - The optical lever arm.

c - Concentration in g/ml^{-1}

c_m - Concentration of protein at the air-liquid meniscus.

c_b - Concentration of protein at the bottom of the solution.

c° - Initial concentration

D - Diffusion coefficient.

f - Frictional coefficient.

L - Phenomenological coefficient.

M_o - Magnification of the camera lens (radial).

M_c - Magnification of the cylindrical lens (height).

N_{Av} - Avagadro's number.

n - Total refractive index.

n_o - Refractive index due to solvent.

Δn - Refractive index due to solute.

R^{sp} - Specific refractive increment.

R - Gas constant (8.314×10^7 ergs deg^{-1} $mole^{-1}$).

r - Distance from the center of rotation.

r_b - Distance from the center of rotation to the bottom of the solution.

r_m - Distance from the center of rotation to the air-liquid meniscus.

r_H - Distance from the center of rotation to the maximum ordinate of the gradient curve.

s - Sedimentation coefficient.

T - Absolute temperature.

t - Time.

$\bar{v}$ - Partial specific volume of solute.

$X = M_o r$

$X_b = M_o r_b$

$X_m = M_o r_m$

ΔX - Radial distance between measurements on the photographic plate.

ΔY - Vertical distance between the solvent and solution pattern.

y - Activity coefficient on the concentration scale.

β - Scheraga-Manderkern constant.

η - Viscosity.

$[\eta]$ - Intrinsic viscosity.

θ - Angle of the schlieren diaphragm.

μ - Chemical potential per gram.

ρ - Density.

ϕ - Sector angle of the cell.

ω - Angluar velocity of the rotor (radians sec^{-1})

Sedimentation-Equilibrium

Theoretical

At equilibrium, the total potential of each component is independent of position in a centrifugal field;

$$\frac{d\mu_i^{total}}{d\ r} = 0. \qquad (1)$$

The force acting on any mass in the positive r direction is $\omega^2 r$. Choosing the point at $r = 0$ as an arbitrary reference for potential energy (PE) and since PE decreases as the particle moves in the positive r direction,

$$PE = -m\int_0^r \omega^2\ r\ d\ r = -m\frac{\omega^2 r^2}{2}, \qquad (2)$$

where m is the particle mass. Since the total potential is the sum of the chemical potential and the centrifugal potential,

$$\mu_i^{total} = \mu_i - \frac{\omega^2 r^2}{2}, \qquad (3)$$

where the potentials are expressed in terms of per gram of component. For a system at equilibrium -- therefore at constant temperature, --

the chemical potential of component i is a function of the pressure and the concentrations of the q components, i.e.,

$$\mu_i = f\ (P,\ c_k) \quad (k = 1,\ \ldots,\ q)$$

Thus, by the laws of differential calculus;

$$d\mu i = \left(\frac{\partial \mu_i}{\partial P}\right)_c d\ P + \sum_{k=1}^{q} \left(\frac{\partial \mu_i}{\partial c_k}\right)_{\substack{T,P,c_j \\ (j \neq o,k)}} d\ c_k. \qquad (4)$$

Substitution of equations (3) and (4) into (1) gives:

$$\left(\frac{\partial \mu_i}{\partial P}\right)_c \frac{dP}{dr} + \sum_{k=1}^{q} \left(\frac{\partial \mu_i}{\partial c_k}\right)_{\substack{T,P,c_j \\ (j \neq o,k)}} \frac{dc_k}{dr} - \omega^2\ r = 0. \qquad (5)$$

But, for an imcompressible system

$$\left(\frac{\partial \mu_i}{\partial P}\right)_c = \bar{v}_i. \qquad (6)$$

Also the pressure varies with the position in the field and may be expressed as

$$\frac{d\ P}{dr} = \omega^2\ r\rho\ . \qquad (7)$$

Incorporating equations (6) and (7) into equation (5) gives

$$\bar{v}_i\ \omega^2\ r\rho + \sum_{k=1}^{q} \left(\frac{\partial \mu_i}{\partial c_k}\right)_{\substack{T,P,c_j \\ (j \neq o,k)}} \frac{dc_k}{dr} - \omega^2\ r = 0. \qquad (8)$$

But the chemical potential <u>per gram</u> of a non-electrolyte varies with the concentration as,

$$\mu_i = \mu_i^o + \frac{R\ T}{M_i}\ \ln\ y_i\ c_i \qquad (9)$$

Thus,

$$\left(\frac{\partial \mu_i}{\partial c_k}\right)_{\substack{T,P,c_j \\ (j \neq o,k)}} = \frac{R\ T}{M_i} \left(\frac{\partial \ln\ y_i}{\partial c^k}\right)_{\substack{T,P,c_j \\ (j \neq o,k)}} \qquad (10)$$

and

$$\left(\frac{\partial \mu_i}{\partial c_i}\right)_{\substack{T,P,c_k \\ (k \neq o,i)}} = \frac{R\,T}{M_i}\left(\frac{\partial (\ln y_i\, c_i)}{\partial c_i}\right)_{T,P,c_k}$$

$$= \frac{R\,T}{M_i}\left(\frac{\partial \ln y_i}{\partial c_i}\right)_{T,P,c_k} + \frac{R\,T}{M_i\, c_i}$$

$$= \frac{R\,T}{M_i\, c_i}\left[1 + c_i \left(\frac{\partial \ln y_i}{\partial c_i}\right)_{\substack{T,P,c_k \\ (k \neq o,i)}}\right]. \qquad (11)$$

the combination of (8), (10), and (11) gives

$$M_i\, \bar{v}_i\, \omega^2\, r\rho + \frac{R\,T}{c_i}\,\frac{dc_i}{dr} + R\,T\left(\frac{\partial \ln y_i}{\partial c_i}\right)_{\substack{T,P,c_k \\ (k \neq o,i)}}\frac{dc_i}{dr}$$

$$+ R\,T \sum_{k=1}^{q}\left(\frac{\partial \ln y_i}{\partial c_k}\right)_{\substack{T,P,c_j \\ (j \neq o,k)}}\frac{dc_k}{dr} - M_i\omega^2\, r = 0 \quad (i \neq k)$$

or

$$M_i\, \bar{v}_i\, \omega^2\, r\rho + \frac{R\,T}{c_i}\,\frac{dc_i}{d\,r} + R\,T \sum_{k=1}^{q}\left(\frac{\partial \ln y_i}{\partial c_k}\right)_{T,P,c_j}\frac{dc_k}{dr}$$

$$- M_i\omega^2\, r = 0 \quad (i = 1, \ldots., q). \qquad (12)$$

Rearranging gives

$$\frac{1}{c_i}\,\frac{dc_i}{dr} + \sum_{k=1}^{q}\frac{\partial \ln y_i}{\partial c_k}\,\frac{dc_k}{dr} = \frac{M_i\,(1 - \bar{v}_i\rho\omega^2\, r}{R\,T}. \qquad (13)$$

For a two component system, equation (13) reduces to

$$\frac{1}{c}\,\frac{d\,c}{d\,r} + \left(\frac{\partial \ln y}{\partial c}\right)_{T,P}\frac{d\,c}{d\,r} = \frac{M\,(1 - \bar{v}\rho\omega^2\, r}{R\,T}$$

or

$$\frac{1}{c}\,\frac{d\,c}{d\,r} = \frac{Mr}{A\left[1 + c\left(\frac{\partial \ln y}{\partial c}\right)_{T,P}\right]} \qquad (14)$$

where

$$A = \frac{R\,T}{(1 - \bar{v}\rho)\omega^2}\,.$$

Experimental Application

The quantitative observation made from the photographic plates is usually the refractive index gradient versus the distance from the origin (schlieren optics). Fortunately, the refractive index ($\underline{n}$) is related to the concentration by the equation;

$$n = n_o + \sum_{i=1}^{q} R_i^{sp} \; c_i \quad \text{and} \tag{15}$$

neglecting c^2 terms which are negligible in most cases. Since $\underline{n} \equiv \underline{n} - \underline{n}_o$ and assuming an incompressible system,

$$\frac{\partial \Delta_n}{\partial r} = \sum_{i=1}^{q} R_i^{sp} \frac{\partial c_i}{\partial r},$$

where R_i^{sp} is the specific refractive increment.

Various procedures have been reported for the calculation of molecular weights of solutes from equilibrium sedimentation data. These procedures are classified here in terms of the type of average molecular weight obtained when the system is polydisperse. In this section, the equations are developed for ideal, monodisperse systems, therefore the molecular weight is defined as an <u>apparent molecular weight</u> ($\underline{M}^{app}$). The true molecular weight is obtained by some suitable extrapolation of $\underline{M}^{app}$ to zero concentration. All of these methods should give the same value for the calculated molecular weight for homogeneous solutes.

<u>Weight-average molecular weight for the entire cell contents</u>. The total solute content of the cell is given by

$$\phi \int_{r_m}^{r_b} cr \, dr \, .$$

Applying the principle of conservation of mass, the total solute content at any time must equal the amount initially placed in the cell, i.e.,

$$\phi \int_{r_m}^{r_b} cr\, dr = \phi\, c^\circ \int_{r_m}^{r_b} r dr = \phi\, \frac{c^\circ}{2} \left(r_b^2 - r_m^2\right)$$

or

$$\int_{r_m}^{r_b} cr\, dr = \frac{c^\circ}{2} (r_b^2 - r_m^2). \qquad (17)$$

Rearranging equation (14) and integrating between $\underline{r}_m$ and $\underline{r}_b$ gives

$$\int_{r_m}^{r_b} cr\, dr = \frac{A}{M^{app}} \int_{c_m}^{c_b} dc = \frac{c^\circ}{2} (r_b^2 - r_m^2)$$

or

$$M^{app} = \frac{2A}{r_b^2 - r_m^2} \cdot \frac{c_b - c_m}{c^\circ} \qquad (18)$$

In these experiments, $\underline{c}_b - \underline{c}_m$ was obtained from the refractive index gradient curve and $\underline{c}^\circ$ from the curve obtained with a synthetic boundary cell. Thus,

$$c_b - c_m = \int_{r_m}^{r_b} \frac{\partial c}{\partial r}\, dr \qquad \text{and}$$

$$c^\circ = \int_{r_m}^{r_p} \frac{\partial c}{\partial r}\, dr. \qquad \text{Using equation (16)}$$

these become

$$c_b - c_m = \frac{1}{R^{sp}} \int_{r_m}^{r_b} \frac{\partial \Delta n}{\partial r}\, dr \qquad (19) \text{ and}$$

$$c^\circ = \frac{1}{R^{sp}} \int_{r_m}^{r_p} \frac{\partial \Delta n}{\partial r}\, dr. \qquad (20)$$

Since

$$\frac{\partial \Delta n}{\partial r} = \frac{\tan \theta}{a' b' M_c} \Delta Y \quad (21) \quad \text{and} \quad r = \frac{X}{M_o} \qquad (22),$$

equation (18) becomes

$$M^{app} = \frac{2\,A}{r_b^2 - r_m^2} \cdot \frac{\frac{1}{R^{sp}} \frac{\Delta X \tan\theta}{a'b'\,M_c\,M_o} \sum_{X_m}^{X_b} \Delta Y \text{ equil.}}{\frac{1}{R^{sp}} \frac{\Delta X \tan\theta}{a'b'\,M_c\,M_o} \sum_{X_m}^{X_p} \Delta Y \text{ syn. boundary}} \qquad (23)$$

If the same schlieren diaphragm angle is used for both photographs, this equation simplifies to

$$M^{app} = \frac{2\,A}{r_b^2 - r_m^2} \cdot \frac{\frac{\Delta X}{M_o} \sum_{X_m}^{X_b} \Delta Y \text{ equil}}{\frac{\Delta X}{M_o} \sum_{X_m}^{X_p} \Delta Y \text{ syn. boundary}} \qquad (24)$$

where $r_b = \frac{X_b}{M_o}$ and $r_m = \frac{X_m}{M_o}$.

Weight-average molecular weight at r (Van Holde - Baldwin plot).

Equation (14) is used directly as $\frac{1}{r}\frac{dc}{dr} = \frac{M^{app}\,c}{A}$. Although c cannot be evaluated directly, it is readily obtained relative to the concentration at the meniscus, r_m. Thus, by adding and subtracting c_m from c

$$\frac{1}{r}\frac{dc}{dr} = \frac{M^{app}}{A}\left[(c - c_m) + c_m\right]. \qquad (25)$$

Applying equations (16), (21), and (22) as before, the following expressions are derived;

$$\frac{1}{X/M_o} \cdot \frac{\tan\theta}{R^{sp}\,a'b'\,M_c} \Delta Y = \frac{M^{app}}{A} \cdot \frac{\Delta X \tan\theta}{R^{sp}\,a'b'\,M_c M_o} \sum_{X_m}^{X} \Delta Y + \text{constant}$$

or

$$\frac{\Delta Y}{X} = \frac{M^{app}}{A\,M_o} \cdot \frac{\Delta X}{M_o} \sum_{Xm}^{X} \Delta Y + \text{constant}. \qquad (26)$$

Therefore $\frac{\Delta Y}{X}$ was plotted against $\frac{\Delta X}{M_o} \sum_{X_m}^{X} \Delta Y$ and M^{app} was obtained from the slope since

$$M^{app} = A\, M_o\, \text{Slope}. \qquad (27)$$

<u>z-Average molecular weight for the entire cell contents.</u> This method utilizes information derived from the intersections of the plot described in the above paragraph with the meniscus and the cell bottom. From equation (14), the following expression is developed:

$$\frac{\frac{1}{r_b}\left(\frac{dc}{dr}\right)_b - \frac{1}{r_m}\left(\frac{dc}{dr}\right)_m}{c_b - c_m} = \frac{M^{app}}{A}. \qquad (28)$$

Applying equations (16), (19), (21), and (22) the above equation becomes:

$$\frac{\frac{1}{r_b}\,\frac{\tan\theta}{R^{sp}a'b'M_c}\Delta Y_b - \frac{1}{r_m}\cdot\frac{\tan\theta}{R^{sp}a'b'M_c}\Delta Y_m}{\frac{\Delta X \tan\theta}{R^{sp}\, a'b'\, M_c\, M_o}\sum_{X_m}^{X_b} \Delta\, Y\ \text{equil}} = \frac{M^{app}}{A}$$

or

$$M^{app} = A\,\frac{\Delta Y_b/r_b - \Delta Y_m/r_m}{\frac{\Delta X}{M_o}\sum_{X_m}^{X_b} \Delta Y\ \text{equil}}. \qquad (29)$$

Example Calculation

The microcomparator readings and the calculations for an equilibrium pattern are presented in this section. The symbol <u>Rn</u> refers to the distance from the inner reference line to the designated position as measured by the microcomparator on the photographic plate. For the

Model E used, r_{inner} = 5.70 cm at low speeds and M_o = 2.103.

Appendix Table 2

Data and example calculation from a short-column equilibrium pattern

Protein: Reduced k-Casein Speed: 14,290 RPM Conc.: 3.2 mg/ml

Temperature: 25.0° C Buffer: 67% acetic acid - 0.15 M NaCl ~~θ:~~ 60°

n	Rn	Xn Rn + 5.70 Mo	ΔYn	$\sum\Delta Yn$	$\Delta Yn/Xn$ X10^3	$\frac{\Delta X}{M_o}\sum\Delta YnX10^3$
0	2.025		0.110*			
1	2.030		0.112*	0.112		
2	2.040		0.114*	0.226		
3	2.050		0.117*	0.343		
4	2.060		0.120*	0.463		
5	2.070		0.123*	0.586		
6	2.080		0.126*	0.712		
7	2.090		0.128*	0.840		
8	2.100	14.087	0.1312	0.9712	9.31	4.62
9	2.110	14.097	0.1322	1.1034	9.38	5.25
10	2.120	14.107	0.1356	1.2390	9.61	5.89
11	2.130	14.117	0.1426	1.3816	10.10	6.57
12	2.140	14.127	0.1434	1.5250	10.15	7.25
13	2.150	14.137	0.1464	1.6714	10.36	7.95
14	2.160	14.147	0.1484	1.8208	10.56	8.66
15	2.170	14.157	0.1526	1.9734	10.78	9.38
16	2.180	14.167	0.1534	2.1268	10.83	10.11
17	2.190	14.177	0.1560	2.2828	11.00	10.85
18	2.200	14.187	0.1584	2.4412	11.17	11.61
19	2.210	14.197	0.1622	2.6034	11.42	12.38
20	2.220	14.207	0.1650	2.7684	11.61	13.16
21	2.230	14.217	0.1658	2.9342	11.66	13.95
22	2.240	14.227	0.1694	3.1036	11.91	14.76
23	2.240	14.237	0.1746	3.2782	12.26	15.59
24	2.260	14.247	0.1762	3.4544	12.37	16.43
25	2.270	14.257	0.1794	3.6338	12.58	17.28
26	2.280	14.267	0.1818	3.8156	12.74	18.14
27	2.290	14.277	0.1834	3.9990	12.85	19.02
28	2.300	14.287	0.1868	4.1858	13.07	19.90
29	2.310	14.297	0.1900	4.3758	13.29	20.81
30	2.320	14.307	0.1940	4.5698	13.56	21.73
31	2.330	14.317	0.1972	4.7670	13.77	22.67
32	2.340	14.327	0.2030	4.9700	14.17	23.63
33	2.350	14.337	0.2068	5.1768	14.42	24.61
34	2.360	14.347	0.2104	5.3872	14.67	25.62
35	2.370	14.357	0.2132	5.6004	14.85	26.63

(Continuation of Table 2)

36	2.380	14.367	0.2178	5.8182	15.16	27.67
37	2.390	14.377	0.2210	6.0392	15.37	28.72
38	2.400	14.387	0.2240	6.2632	15.57	29.78
39	2.410		0.228*	6.4912		
40	2.420		0.232*	6.7232		
41	2.430		0.236*	6.9592		
42	2.440		0.239*	7.1982		
43	2.4465		0.241*			

$R_b = 2.448$ $\quad r_b = \frac{X_b}{M_o} = 6.864$ $\quad \Delta Y_m = 0.110$

$R_m = 2.025$ $\quad \Delta Y_b = 0.242$

$r_m = \frac{X_m}{M_o} = 6.663$

---

* Values obtained by extrapolation.

$$c_b - c_m = \frac{\Delta X}{M_o} \sum_{1}^{42} \Delta Y_n + \frac{R_b - (R_{42} + \frac{\Delta X}{2})}{M_o} \Delta Y \left[R_{42} + \frac{\Delta X}{2} + \left(\frac{R_b - (R_{42} + \frac{\Delta X}{2})}{2} \right) \right]$$

$$c_b - c_m = \frac{0.01}{2.103}(7.1982) + \frac{0.003}{2.103}(0.241) = 0.03457.$$

Note that the first value for ΔY used in the summation was obtained at $X_m + \frac{\Delta X}{2}$ and similarly, the ΔY was obtained at the midpoint of the small interval remaining at the bottom. Essentially, this method represents trapezoidal integration. C_o is obtained by similar measurements of a synthetic boundary pattern obtained at the same schlieren diaphragm angle, ie.

$$c_o = \frac{\Delta X}{M_o} \sum_{X_m}^{X_p} \Delta Y_{\text{syn. boundary}} = 0.04904 \quad \text{for this case.}$$

Other quantities necessary for the calculations are a) $\rho_{\text{solution}} = 1.0688$, b) $\frac{RT}{(1 - \bar{v}\rho)\omega^2} = A = \frac{(8.314 \times 10^7)(298)}{(1 - 0.73 \times 1.0688)(2.23936 \times 10^6)} = 50{,}290$, and c) $\frac{1}{r_b^2 - r_m^2} = 0.368$. The different average molecular weights are obtained of follows:

a) weight-average molecular weight for the total cell contents; from equation (24)

$$M_w^{app} = (2)\ (50,290)\ (0,368)\ \frac{0.03457}{0.04904} \approx 26,100$$

b) z-average molecular weight for the total cell contents; from equation (29)

$$M_z^{app} = 50,290\ \frac{0.242/6.864 - 0.110/6.663}{0.03457} \approx 27,260$$

c) weight-average molecular weight from the Van Holde-Baldwin plot; from equation (26). The slope is obtained from a plot of $\Delta Y_n/X \times 10^3$ against $\frac{\Delta X}{M_o}\sum \Delta Y_n \times 10^3$, then $M^{app} = M_o A$ Slope = (2.103) (50,290)(0.242) = 25,600.

Calculation of the Diffusion Coefficient

The diffusion coefficient can be obtained by combining measurements of photographs taken after the plateau region disappears from the sedimentation-equilibrium pattern. The simplest method for such calculations is that proposed by Sophianopoulos *et al.* (1962) utilizing the following equation;

$$\ln\left[\left(\frac{\partial c}{\partial r}\right)_{eq} - \left(\frac{\partial c}{\partial r}\right)_t\right]_{\text{mid point}} = \text{constant} - \frac{\pi^2 D}{r_b^2 - r_m^2} A_I\ A_{II}\ t, \quad (30)$$

where

$$A_I = 1 + \frac{3}{\pi^2}\left(\frac{r_b - r_m}{r_b + r_m}\right)^2 \qquad \text{and}$$

$$A_{II} = 1 + \frac{(r_b - r_m)^2}{2}\left(\frac{\omega^2 M (1 - \bar{v}\rho)}{2 RT}\right)\left(\frac{r_b - r_m}{2}\right)^2 .$$

By removing the optical constants, equation (30) becomes

$$\ln \left(\Delta Y_{eq} - \Delta Y_t \right)_{\text{mid point}} = \text{constant}' - \frac{\pi^2 D}{r_b^2 - r_m^2} A_I A_{II} \, t \, . \quad (31)$$

Thus, log $(\Delta Y_{eq} - \Delta Y_t)$ measured at the mid point of the solution column was plotted against the time (min), letting the first photograph be zero time since only the time interval is important. The slope of this plot is given by

$$\text{slope} = \frac{-\pi^2 D \; A_I \, A_{II}}{2.303 \, (r_b - r_m)^2} \, .$$

Changing the diffusion coefficient from $cm^2 \; min^{-1}$ to $cm^2 \; sec^{-1}$, then, gives

$$D = - \frac{2.303 \, (r_b - r_m)^2 \, (\text{slope})}{\pi^2 \, A_I \, A_{II} \, 60} \; cm^2 \; sec^{-1} \, . \quad (32)$$

Approach-to-Equilibrium

Theoretical

The flow equation for the ultracentrifuge has been derived from the laws of irreversible thermodynamics and for incompressible systems is

$$J_i = - \sum_{k=1}^{q} L_{ik} \left(\frac{\partial \mu_k^{\text{total}}}{\partial r} \right)_t \qquad (i = 1, \ldots, q). \quad (33)$$

Differentiating equation (3) with respect to $\underline{r}$ at constant time gives

$$\left(\frac{\partial \mu_k^{\text{total}}}{\partial r} \right)_t = \left(\frac{\partial \mu_k}{\partial r} \right)_t - \omega^2 r \, .$$

For an isothermal system the chemical potential is a function of the pressure and concentrations of all solutes, therefore

$$\left(\frac{\partial \mu_k}{\partial r}\right)_t = \left(\frac{\partial \mu_k}{\partial P}\right)_{T,c,t}\left(\frac{\partial P}{\partial r}\right)_{T,t} + \sum_{j=1}^{q} \left(\frac{\partial \mu_k}{\partial c_j}\right)_{T,P,c_i}\left(\frac{\partial c_j}{\partial r}\right)_t .$$

Substituting this result along with equations (6) and (7) into equation (33) gives

$$J_i = -\sum_{k=1}^{q} L_{ik}\left[-\omega^2 r\,(1 - \bar{v}_k\rho) + \sum_{j=1}^{q} \left(\frac{\partial \mu_k}{\partial c_j}\right)_{T,P,c_i}\left(\frac{\partial c_j}{\partial r}\right)_t\right] (i = 1, \ldots., q) \quad (34)$$

This equation is converted to the corresponding practical flow equation,

$$J_i = c_i S_i \omega^2 r - \sum_{j=1}^{q} D_{ij}\left(\frac{\partial c_j}{\partial r}\right) \quad (i = 1, \ldots., q) \quad (35)$$

where

$$S_i = \sum_{k=1}^{q} \frac{L_{ik}}{c_i}(1 - \bar{v}_k\rho) \qquad (i = 1, \ldots., q)$$

$$D_{ij} = \sum_{k=1}^{q} L_{ik}\left(\frac{\partial \mu_k}{\partial c_j}\right) \qquad (i = 1, \ldots., q)$$

The Archibald molecular weight can then be obtained by applying the boundary conditions to the centrifuge, and are represented by

$$J_i = 0 \quad (i = 1, \ldots., q) \quad (r = r_m, r_b). \quad (36)$$

For a two component system, equation (34) becomes

$$J = L(1 - \bar{v}\rho)\omega^2 r - L\left(\frac{\partial \mu}{\partial c}\right)_{T,P}\left(\frac{\partial c}{\partial r}\right)_t , \text{ and since } J = 0 \text{ at}$$

$\underline{r} = \underline{r}_m$,

$$(1 - \bar{v}\rho)\omega^2 r_m - \left(\frac{\partial \mu}{\partial c}\right)_{T,P}\left(\frac{\partial c}{\partial r}\right)_m = 0. \tag{37}$$

Substituting in equations (9) and (11) for $\left(\frac{\partial \mu}{\partial c}\right)_{T,P}$ yields

$$(1 - \bar{v}\rho)\omega^2 r_m - \frac{RT}{Mc_m}\left[1 + c\left(\frac{\partial \ln y}{\partial c}\right)_{T,P}\left(\frac{\partial c}{\partial r}\right)_m\right] = 0$$

which gives upon rearranging

$$\frac{\left(\frac{\partial c}{\partial r}\right)_m}{\omega^2 r_m} = \frac{M(1 - \bar{v}\rho)c_m}{RT\left[1 + c\left(\frac{\partial \ln y}{\partial c}\right)_{T,P}\right]}. \tag{38}$$

By adding and subtracting a constant, $\underline{c}^\circ$, from the right-hand side of equation (38), one obtains

$$\frac{\left(\frac{\partial c}{\partial r}\right)_m}{\omega^2 r_m} = \frac{M(1 - \bar{v}\rho)\left[(c_m - c^\circ) + c^\circ\right]}{RT\left[1 + c\left(\frac{\partial \ln y}{\partial c}\right)_{T,P}\right]} \tag{39}$$

where $c_m - c^\circ = -\frac{1}{r_m^2}\int_{rm}^{r} r^2 \frac{\partial c}{\partial r}\, dr$. (40)

Experimental Application

Rewriting equation (39) in terms of the refractive index and rearranging signs gives

$$\frac{\frac{1}{R^{sp}}\left(\frac{\partial \Delta n}{\partial r}\right)_m}{\omega^2 r_m} = -\frac{M(1 - \bar{v}\rho)\left[(c^\circ + c_m) - c^\circ\right]}{RT\left[1 + c\frac{\partial \ln y}{\partial c}_{T,P}\right]}. \tag{41}$$

The quantity $(c^\circ - c_m)$ may be evaluated by several methods, however, the method using the Trautman z-scale is the most direct. Thus, a quantity is defined

$$Z \equiv (10X / X_r)^3 \qquad (42),$$

where X_r is an arbitrary reference point beyond the cell bottom, chosen here as 160 mm. This gives

$$X^2\, dX = (X_r^3/3000)\, d\, Z \qquad (43)$$

upon differentiation. Since

$$c^\circ - c_m = \frac{1}{R^{sp}\, r_m^2} \int_{rm}^{r} r^2 \frac{\partial \Delta n}{\partial r}\, dr =$$

$$\frac{\tan\theta}{R^{sp}\, X_m^2\, a'b'\, M_c M_o} \int_{X_m}^{X} X^2\, \Delta Y\, dX\,,$$

use of equation (43) gives

$$c^\circ - c_m = \frac{X_r^3 \tan\theta}{R^{sp}\, X_m^2\, a'b'\, M_c\, 3000\, M_o} \int_{Z_m}^{Z} \Delta Y\, dZ$$

or

$$c^\circ - c_m = \frac{F_x}{a'b'M_c} \left[\frac{X_r}{X_m}\right]^2 \frac{\Delta Z \tan\theta}{R^{sp}} \sum_{j=1}^{n} \Delta Y_j \qquad (44)$$

where

$$F_x \equiv X_r/3000\, M_o\,. \qquad (45)$$

Applying these results to equation (39) one obtains

$$\frac{\frac{1}{R^{sp}} \frac{\tan\theta}{a'b'\, M_c}\, \Delta Y_m}{\omega^2\, r_m} = -\, M^{app}\, \frac{(1 - \bar{v}\rho)}{RT} \frac{F_x}{a'b'M_c} \left[\frac{X_r}{X_m}\right]^2 \frac{\Delta Z \tan\theta}{R^{sp}} \sum_{j=1}^{n} \Delta Y_j$$

where

$$M^{app} = M/1 + c\left(\frac{\partial \ln y}{\partial c}\right)_{T,P} + \text{constant}\,.$$

Multiplying through by the optical constants and using a constant θ yields

$$\frac{\Delta Y_m}{\omega^2 r_m} = -M^{app} \frac{(1 - \bar{v}\rho)}{RT} F_x \left[\frac{X_r}{X_m}\right]^2 \Delta Z \sum_{j=1}^{n} \Delta Y_j + \text{constant.} \quad (46)$$

M^{app} can then be obtained from the slope of a plot of $\Delta Y_m/\omega^2 r_m$ against $F_x \left[\frac{X_r}{X_m}\right]^2 \Delta Z \sum_{j=1}^{n} \Delta Y_j$.

(Note: In Part II of this thesis, these quantities were denoted as $\left(\frac{dn}{dx}\right)_m / \omega^2 X_m$ and $c^\circ - c_m$).

To obtain the greatest accuracy, at least three widely separated rotor speeds should be used. In the case of a paucidisperse system, a change in slope occurs when all of the heavy components have sedimented away from the meniscus, thus allowing the molecular weight of the light component to be determined.

Sedimentation-Velocity

Calculation of the Coefficient

The observed sedimentation coefficient. The sedimentation coefficient is defined as the sedimenting velocity (u) per unit of centrifugal field. Thus

$S \equiv \frac{u}{\omega^2 r}$. (47). The velocity corresponds to the movement of particles in the plateau region (where $\partial c/\partial r = 0$) and it can be shown that the true sedimentation-velocity is given by the rate of movement of the square-root of the second movement of the refractive index gradient curve (r_z). However, the position of r_z is equivalent

to the position of the maximum ordinate (r_H) for symmetrical boundaries. Therefore,

$$S = \frac{dr_H}{dt} \Big/ \omega^2 r_H \qquad \text{which upon integration gives}$$

$$\ln r_H = \omega^2 S t + \text{constant} \qquad \text{or}$$

$$\log \frac{X_H}{100} = \frac{\omega^2 S}{2.303} t + \text{constant}' \qquad (48)$$

where X_H is in mm. For the purpose of making the calculations, $\log \frac{X_H}{100}$ is plotted against time (min) and the sedimentation coefficient obtained from the slope of the plot.

Correction of the observed S to standard conditions. The experimentally determined sedimentation was corrected to a value corresponding to sedimentation in water at 20° C. The equation commonly used is

$$S_{20,w} = \left(\frac{\eta_{w,t}}{\eta_{w,20}}\right)\left(\frac{\eta_{o,t}}{\eta_{w,t}}\right)\left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{t,o}}\right) S_T, \qquad (49)$$

where the first term is the viscosity of water at the experimental temperature relative to that at 20° C, the second term is the relative viscosity of the solvent to that of water, and the last term is the relative buoyancy term containing the partial specific volume, the density of water at 20° C, and the density of the solution at the experimental temperature.

Correction for radial dilution. Since the cell is sector-shaped, the concentration in the plateau region is constantly changing. This factor must be considered when the values obtained at finite concentrations are extrapolated to infinite dilution to obtain $S^\circ_{20,w}$. If the slope of the plot described in the first paragraph is obtained by the

method of least-squares, the actual concentration corresponding to the observed coefficient is the average of the concentrations for the first and last frame used for the calculation. The initial concentration (c°) placed in the cell and the concentration in the plateau region (c_p) at any other time are related by the equation

$$\frac{c_p}{c^\circ} = \left(\frac{r_m}{r_H}\right)^2 \tag{50}$$

or more simply

$$\frac{c_p}{c^\circ} = \left(\frac{X_m}{X_H}\right)^2 . \tag{51}$$

Therefore, if the initial concentration is known, the concentrations for the first and last frame may be obtained.

The radial dilution rule also provides a method of checking the homogeneity of the sample since the area under the curve corrected to radial dilution by equation (51) should be constant.

Relation of the Sedimentation Coefficient to Particle Shape

The frictional ratio. The frictional coefficient for a rigid, uncharged sphere is given by Stokes law

$$f = 6\pi\eta r, \tag{52}$$

where r is the radius of the sphere. Assuming no solvation, the radius of a molecule is given by $r = \left(\frac{3}{4\pi}\frac{M\bar{v}}{N_{AV}}\right)^{1/3}$. Therefore the minimum possible frictional coefficient per mole of solute (fo) would be

$$fo = 6\pi\eta\left(\frac{3}{4\pi}\frac{M\bar{v}}{N_{AV}}\right)^{1/3} \approx 1.39 \times 10^{-9} \left(M\,\bar{v}_{20,w}\right)^{1/3} . \tag{53}$$

Equation (35) defined the sedimentation coefficient of a particle as

$$S_1 = \frac{L_{11}}{c_1}\ (1 - \bar{v}_1\rho) \qquad \text{and the diffusion coefficient as}$$

$$D_{11} = L_{11}\left(\frac{\partial \mu_1}{\partial c_1}\right)_{T,P} \qquad \text{for a two-component system. Since}$$

$$\mu_1 = \mu_1^\circ + \frac{RT}{M_1} \ln y_1 c_1, \quad D_{11} = L_{11}\frac{RT}{M_1 c_1}\left[1 + c_1\left(\frac{\partial \ln y_1}{\partial c_1}\right)\right] = \frac{RT}{N_{AV} f_1};$$

where f_1 is the frictional coefficient per mole. Thus

$$L_{11} = \frac{M_1 c_1}{N_{AV} f_1}\ \frac{1}{1 + c_1 \dfrac{\partial \ln y_1}{\partial c_1}} \qquad \text{and}$$

$$S_1 = \frac{M_1 (1-\bar{v}_1\rho)}{N_{AV} f_1 \left[1 + c_1 \dfrac{\partial \ln y_1}{\partial c_1}\right]} \qquad \text{or in the limit,}$$

$$c_1 \longrightarrow 0, \quad S^\circ_{20,w} = \frac{M_1 (1 - \bar{v}_{20,w}\rho_{20,w})}{N_{A_V} f_1}\ . \tag{54}$$

Therefore,

$$f/f_o = \frac{\dfrac{M (1 - \bar{v}_{20,w}\rho_{20,w})}{N_{A_V} S^\circ_{20,w}}}{1.39 \times 10^{-9}\ (M\bar{v}_{20,w})}^{1/3}\ . \tag{55}$$

This ratio has been related to the axial ratios of prolate and oblate ellipsoids by use of Perrin's equation. These ratios are tabulated in many of the references given previously.

The Scheraga-Mandelkern constant. According to Scheraga and Mandelkern (1953), the intrinsic viscosity is related to the effective hydrodynamic volume, V_e, of the particle, thus

$$[\eta]\ (\mathrm{dl/g}) = \frac{N_{AV}}{100}\ \frac{Ve}{M}\ \nu \tag{56}$$

where ν is a shape factor. Note that $[\eta]$ is in dl/g ($[\eta]$ in ml/g = 100 $[\eta]$ in dl/g). Since hydrodynamic properties depend on both the particle size and shape, these authors pointed out that two hydrodynamic parameters must be obtained to define the size and shape.

The frictional coefficient for a sphere having a volume equal to Ve is

$$f_o = 6 \pi \eta a_o . \tag{57}$$

Substituting this equation into equation (54) gives

$$S^\circ_{20,w} = \frac{M (1 - \bar{v}_{20,w} \rho_{20,w})}{N_{Av} f/f_o 6 \pi \eta_{20,w} a_o} . \tag{58}$$

But $a_o = \left(\frac{3 V_e}{4 \pi}\right)^{1/3}$ and substituting equation (56) for V_e and rearranging results in the following expression:

$$\frac{M (1 - \bar{v}_{20,w} \rho_{20,w})}{S^\circ_{20,w} N_{Av}} = f/f_o \, 6 \pi \eta_{20,w} \left(\frac{300 M [\eta]}{4 \pi \nu N_{Av}}\right)^{1/3}$$

or

$$f_o/f \left(\frac{N_{Av} \nu}{16200 \pi^2}\right)^{1/3} = \frac{N_{Av} S^\circ_{20,w} [\eta]^{1/3} \eta_{20,w}}{M^{2/3} (1 - \bar{v}_{20,w} \rho_{20,w})} = \beta. \tag{59}$$

Thus, two hydrodynamic properties -- $S^\circ_{20,w}$ and $[\eta]$ -- and a thermodynamically determined molecular weight were used in these studies to calculate β. However, β is not very sensitive to particle shape and therefore the effective dimensions of the molecules were not calculated. Since β is not very sensitive to particle shape, an approximate molecular weight can be calculated from the intrinsic viscosity and sedimentation coefficient. Assuming an average value for β (2.16×10^6) and substituting the value for the viscosity of water at 20° C in equation (59) gives

$$M = \frac{4690\ (S^{\circ}_{20,w})^{3/2}\ [\eta]^{1/2}}{(1 - \bar{v}_{20,w}\ \rho_{20,w})} \quad . \qquad (60)$$

APPENDIX III

Appendix Table 3

A comparison of the amino acid analysis for k-casein and the λ-casein fraction

Amino Acid	k-Casein g/100 g	λ-Casein Fraction g/100 g
Asp	7.72	8.18
Thr	6.74[a]	1.65[a]
Ser	5.03[a]	3.17[a]
Glu	19.80	24.11
Pro	10.95	7.16
Gly	1.23	2.63
Ala	5.40	2.86
Val	6.30	5.89
Met	1.68	1.99
Ilen	7.10	5.18
Leu	6.11	8.23
Tyr	7.61	5.67[a]
Phe	3.86	4.55
Lys	6.51	9.04
His	2.36	3.83
Arg	3.96	4.17
NH_3	1.94[a]	1.90

[a] Extrapolated to zero hydrolysis time.