Chromatographic Isolation and Partial Characterization of Reduced κ-Casein Components*

J. H. Woychik, E. B. Kalan, and M. E. Noelken

ABSTRACT: κ-Casein of types A and B were prepared from the milks of individual cows. The κ-casein was reduced and alkylated, allowing the chromatographic separation from each type of a single major component and several minor components. The major components, designated A-1 and B-1, have molecular weights of about 19,000 and appear to be free of carbohydrate. Amino acid data reveal that component A-1 has one residue each of aspartic acid and threonine more than component B-1, which has one residue each of alanine and isoleucine more than component A-1. The components, whether containing carbohydrate or not, maintain their ability to stabilize α_{s1}-casein and their rennin sensitivity.

The amino acid composition of several of the minor components from the two types of κ-casein is similar to, but not identical with, the major component with which the minor component is associated.

Polymorphism has been demonstrated in κ-casein by gel electrophoresis of the reduced protein (Neelin, 1964; Schmidt, 1964; Woychik, 1964). This polymorphism is based on the occurrence of two major components, either singly or in pairs, in the reduced κ-caseins obtained from the milk of individual cows. Additional heterogeneity was indicated by the presence of several minor electrophoretic components (Mackinlay and Wake, 1964; Schmidt, 1964; Woychik, 1964) in the reduced κ-caseins. Further studies of the occurrence of this polymorphism among individual cows and in different breeds should eventually establish whether the observed polymorphism is under genetic control. Preliminary evidence from such studies from several laboratories (Schmidt, 1964; Woychik, 1965a) support the idea of a genetic control.

The absence of electrophoretic resolution in "native" κ-casein has been attributed at least partly to the occurrence of randomly formed intermolecular disulfide bonds (Mackinlay and Wake, 1964; Woychik, 1965b). These intermolecular bonds could lead to the formation of a polydisperse material. This material, upon gel electrophoresis, produces a broad, unresolved zone arising from the "sieving effect" of the gel pores on the κ-casein aggregates. Whether the major electrophoretic components observed after reduction represent individual κ-casein molecules cannot be determined from the limited information available. However, the retention of the α_{s1}-casein stabilizing ability and rennin sensitivity by the reduced and alkylated κ-caseins (Mackinlay and Wake, 1964, 1965; Woychik, 1965b) at least suggests that the individual components represent both the functional and structural units of κ-casein.

The isolation of the individual components of reduced κ-casein would greatly facilitate structural studies and perhaps permit the elucidation of the mechanisms of casein micelle stabilization and of rennin action. Several attempts have been made to fractionate "native" κ-casein in the presence of concentrated urea (Ribadeau Dumas *et al.*, 1964; Rose and Marier, 1963). Although fractions were obtained which varied in sialic acid and phosphorus content, none of them appeared to be discrete components because of the intermolecular disulfide bonding in the "native" κ-casein.

The present paper describes the chromatographic isolation of the individual components from both alkylated and nonalkylated reduced κ-casein. The amino acid composition and molecular weight of the major components are reported, as well as the amino acid composition of several minor components. The ability of each fraction to stabilize α_{s1}-casein and the rennin sensitivity of each fraction are also reported.[1]

Experimental Methods

Preparation of "Native" and Reduced κ-Caseins. The κ-caseins used in these investigations were of types A and B (Neelin, 1964) and were obtained from the milk of individual cows having only one of the major components. Until the chemistry of κ-casein is better understood and until the genetic control of polymorphism

* From the Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Philadelphia, Pennsylvania 19118. *Received December 2, 1965; revised April 22, 1966.*

[1] While this paper was in preparation, a report (Mackinlay and Wake, 1965) appeared on the fractionation of S-carboxymethyl-κ-casein and characterization of the components. Although the scope of Mackinlay and Wake's investigation was considerably different from ours, such observations on κ-casein components as were made by both groups are in substantial agreement.

of κ-casein is established without question, problems of nomenclature are difficult to avoid. In the present paper we have designated the two types of κ-casein as κ-casein A and κ-casein B; the major component of reduced κ-casein A and reduced κ-casein B as κ-casein A-1 and κ-casein B-1; and the minor components of reduced κ-casein A and reduced κ-casein B, consecutively in the order of increasing electrophoretic mobility under the specified conditions, as A-2, A-3, etc., and B-2, B-3, etc.

κ-Casein was isolated from acid precipitated whole casein by the urea–sulfuric acid method (Zittle and Custer, 1963) and purified by precipitation from ethanolic solution (McKenzie and Wake, 1961). Carboxamidomethyl-κ-casein (CAM-κ)[2] was prepared according to the method previously reported (Woychik and Kalan, 1965). A 2% solution of κ-casein was reduced for 1 hr in 4 M urea–pH 7.0 imidazole buffer (0.01 M) with 2-mercaptoethanol at a molar ratio of 10:1 based on a theoretical sulfhydryl content of 10 moles/100,000 g of protein (Woychik and Kalan, 1965). The reduced κ-casein was then alkylated for 10 min with an excess of iodoacetamide (the molar ratio of iodoacetamide–total sulfhydryl present was 3:1), while maintaining the pH at 7.0. After 10 min of alkylation, the residual iodoacetamide was treated with an excess of 2-mercaptoethanol and the reaction mixture was dialyzed and lyophilized. This method results in the complete reduction of κ-casein and has been shown to be specific for the alkylation of the thiol groups of the reduced protein (Woychik and Kalan, 1965; Kalan and Woychik, 1965).

Chromatography of Reduced κ-Casein. DEAE-cellulose was prepared for chromatographic use by removal of the fines by decantation and washing with 0.5 N sodium hydroxide, water, 0.1 N hydrochloric acid, and water in that order. The washed DEAE-cellulose was suspended in water, titrated to pH 7.0, and finally equilibrated with 0.01 M imidazole buffer, pH 7.0. The chromatographic columns were packed at a pressure of 6 psi. Prior to the application of the protein, the column was equilibrated with 0.01 M imidazole–4 M urea, pH 7.0 (starting buffer).

The κ-casein to be chromatographed was applied to the column as a 2% solution in starting buffer, and then developed at room temperature with 1800 ml of buffer at a constant flow rate of 75 ml/hr using a linear sodium chloride gradient (0.02–0.09 M in starting buffer). The absorbancy of the eluate at 280 mμ was monitored with a Canalco ultraviolet flow analyzer.[3] Aliquots comprising a given fraction were pooled, dialyzed, and lyophilized.

For chromatography of reduced nonalkylated κ-casein, the protein was first reduced with a 10-fold excess of 2-mercaptoethanol for at least 2 hr prior to application to the column. The column was then developed with the identical gradient listed above except that 1.0 ml of 2-mercaptoethanol was added/l. of buffer solution.

Polyacrylamide Gel Electrophoresis. Electrophoretic patterns were obtained for κ-casein and its fractions in pH 9.2 Tris buffer (Peterson, 1963) according to the procedure previously reported (Woychik, 1964). Nonalkylated κ-casein fractions were reduced with 2-mercaptoethanol prior to application to the acrylamide gel.

Sialic Acid and Hexose Determination. Sialic acid determinations were made using the procedure of Warren (1959) after first hydrolyzing the bound sialic acid by incubating the protein in 0.1 N H_2SO_4 for 1 hr at 80°. Crystalline, synthetic *N*-acetylneuraminic acid (Sigma Chemical Co.) was used as a standard. Hexose was determined using the orcinol procedure as reported by Winzler (1955) using galactose as a standard.

α_{s1}-Casein Stabilization and Preparation of p-κ Derivatives. The ability of the isolated CAM-κ components to stabilize α_{s1}-casein in the presence of calcium ions was determined according to the procedure described by Zittle (1961). The *p*-κ derivatives were prepared by digesting 2 mg of the protein with 5 μg of rennin in 0.1 ml of pH 7.0 imidazole buffer (0.005 M). After 5 min of digestion, the rennin was inactivated by the addition of 0.1 ml of pH 9.2 Tris–7 M urea buffer. The CAM-*p*-κ derivatives dissolved readily upon addition of the urea buffer. However, the *p*-κ derivatives obtained from unreduced κ-casein required the addition of a drop of 2-mercaptoethanol to effect solution.

Amino Acid Analysis. The κ-casein components were analyzed for their amino acid compositions by the method of Piez and Morris (1960). About 2 mg of protein was hydrolyzed with glass-distilled 6 N hydrochloric acid in a sealed, evacuated tube. Hydrolyses for each component were done in triplicate for each of the time periods at 110° in a circulating-air laboratory oven. The data reported are in the tables as residues per minimal molecular weight calculated by finding the molar ratios based on eight different amino acids (Asp, Pro, Ala, Met, Leu, Phe, His, and Arg).

Sedimentation Analysis. Sedimentation equilibrium experiments on the two major CAM-κ components A-1 and B-1 were performed at 25° with a Beckman-Spinco Model E ultracentrifuge equipped with interference optics. The technique used to determine the molecular weights was that described by Yphantis (1964). The solvent used was 5 M guanidine hydrochloride–0.05 M potassium acetate–acetic acid (pH 5.3); its density was determined pycnometrically. The partial specific volume of the κ-casein components was assumed to be 0.729 ml/g, the value calculated from the amino acid composition by Swaisgood and Brunner (1963). For the A-1-κ-casein component the initial concentration was 0.015%, the column height was 0.83 cm, and the speed was 37,020 rpm. For the B-1-κ-casein component, the initial concentration was 0.015%, the column height was 0.73 cm, and the speed 42,040 rpm.

[2] Abbreviations used: CAM-κ, carboxamidomethyl-κ-casein; UA and MP, unadsorbed and macropeptide fractions.

[3] It is not implied that the U. S. Department of Agriculture recommends the above-mentioned company or its product to the possible exclusion of others in the same business.

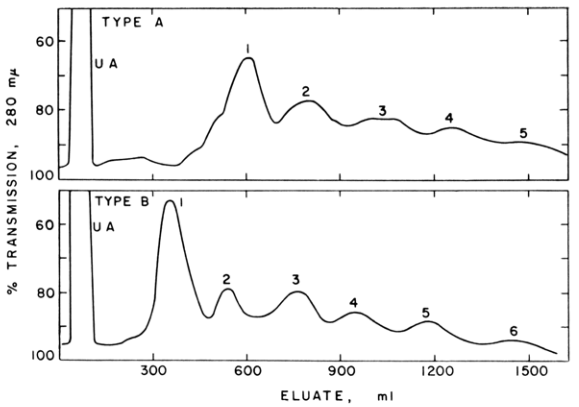

FIGURE 1: Elution curves obtained following chromatography of 250 mg of CAM-κ-caseins A and B from a 2 × 10 cm DEAE-cellulose column.

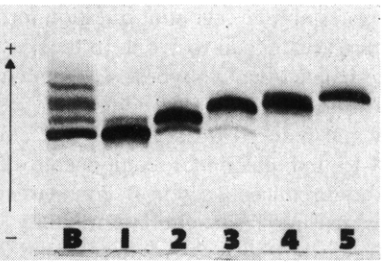

FIGURE 2: Polyacrylamide gel electrophoretic patterns (pH 9.2) of type B CAM-κ-casein and fractions following chromatography on DEAE-cellulose.

Results

Chromatographic Fractionation of CAM-κ-Casein. Previous chromatographic studies of κ-casein have utilized either whole casein or purified κ-casein preparations in which the "native" state of κ-casein was retained. Although various κ-casein fractions were obtained in a fairly broad elution range, none of the fractions differed from one another, as determined by their electrophoretic behavior. The lack of chromatographic and electrophoretic resolution can perhaps be attributed to the varying degrees of aggregation arising from oxidation of sulfhydryl groups leading to the formation of intermolecular disulfide bonds. The effect of disulfide bond reduction on the electrophoretic behavior of κ-casein has now been well demonstrated. This reduction has resulted in the electrophoretic resolution of two major and several minor components. Thus the ability to reduce κ-casein into discrete electrophoretic components and to maintain their monomeric states should greatly facilitate their chromatographic isolation.

Whether reduced κ-casein should be alkylated prior to chromatography will depend upon the expected use of the various fractions; however, in most cases a stable reduced derivative would be preferable. In the selection of an alkylating agent for the preparation of reduced and alkylated κ-casein, it was desirable to avoid the incorporation of additional charged groups which may interfere in subsequent studies such as the stabilization of α_{s1}-casein and the action of rennin. Furthermore, an alkylated cysteine derivative amenable to quantitative chromatographic analysis was preferred. These conditions were satisfied by the use of iodoacetamide.

Preliminary experiments with CAM-κ on DEAE-cellulose columns had indicated that all of the components could be eluted with pH 7.0 imidazole (0.01 M)–4 M urea buffer containing 0.1 M sodium chloride. Various elution gradients to 0.1 M sodium chloride were investigated and a linear gradient from 0.02 to 0.09 M sodium chloride in an 1800-ml volume of buffer was finally selected.

The results obtained following the elution of 250 mg of CAM-κ-caseins A and B from 2 × 20 cm DEAE-cellulose columns are shown in Figure 1. Each κ-casein yielded, in addition to the peak containing the major component, four to five lesser peaks and an unadsorbed (UA) fraction. The unadsorbed fraction accounted for approximately 15% of the applied protein and upon gel electrophoresis (not shown) consisted of a major band with a mobility approximately half that of the major B-1 component plus several slower migrating minor components. This fraction had no α_{s1}-casein stabilizing ability or rennin sensitivity and was therefore not investigated further in the present study. The electrophoretic patterns of the principal fractions obtained from the type B CAM-κ are shown in Figure 2 together with the pattern of unfractionated B κ-casein. The degree of component overlapping in these initial fractions is evident from the electrophoretic patterns and is representative of the fraction used for rechromatography.

The electrophoretic components of the type A κ-casein were obtained in a similar degree of purity. Chromatography of reduced nonalkylated κ-casein in the presence of 2-mercaptoethanol yielded comparable elution diagrams and electrophoretic patterns.

Larger amounts of the initial κ-casein fractions were obtained by chromatographing 1 g of CAM-κ on a 2 × 30 cm DEAE-cellulose column and eluting with a linear salt gradient from 0.02 to 0.09 M in a total buffer volume of 3600 ml.

Each protein fraction obtained from the initial chromatography was rechromatographed using the same gradient (0.02–0.09 M sodium chloride in 1800 ml). Between 25 and 50 mg of the fractions were applied to a 2 × 10 cm column and developed at 75 ml/hr. Each of the components eluted at approximately the same molarity at which it was initially obtained. The components obtained following a single rechromatographing showed only trace amounts of the overlapping components. Both major components, A-1 and B-1, were subjected to a second rechromatographing and were found to be electrophoretically homogeneous prior to amino acid analysis.

Stabilization of α_{s1}-Casein and Rennin Sensitivity.

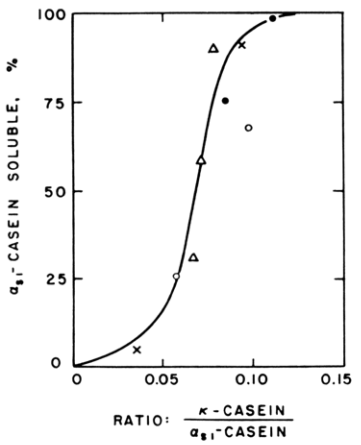

FIGURE 3: Stabilization of α_{s1}-casein by type B CAM-κ-casein components. ○ = B-1, x = B-2, △ = B-3, ● = B-4.

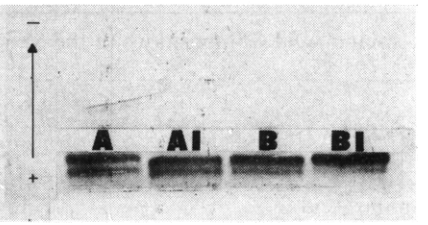

FIGURE 4: Polyacrylamide gel electrophoretic patterns (pH 9.2) of the *p*-κ-caseins of type A and B κ-caseins, and of the isolated principal components A-1 and B-1.

The ability of reduced and alkylated κ-casein to stabilize α_{s1}-casein in the presence of calcium ions has already been reported (Mackinlay and Wake, 1964; Woychik, 1965b). Thus, neither intact disulfide bonds nor free sulfhydryl groups are required for stabilization. Although preliminary results (Woychik, 1964) indicated that the observed stabilization of α_{s1}-casein did not require the combined effect of several of the electrophoretic components, a further study of the stabilizing ability of the isolated components was desirable. The percentage α_{s1}-casein solubilized in the presence of calcium ions at various component to α_{s1}-casein ratios is presented in Figure 3. The curve was drawn to fit the points obtained with the major and four minor components of the type B CAM-κ-casein at different κ/α_{s1}-casein ratios. The figure thus shows that the components have essentially the same ability to solubilize α_{s1}-casein, and that the final curve is comparable to that obtained with whole "native" κ-casein (Zittle, 1961).

The splitting of κ-casein by rennin into the *p*-κ-casein and macropeptide fractions (MP) has been well demonstrated. At pH 9.3, *p*-κ-casein migrates in the direction opposite to that of κ-casein; this can be attributed to the loss of the acidic groups associated with the macropeptide. However, the *p*-κ-casein remains intermolecularly bound by disulfide bonds and is therefore for the most part unable to migrate through the pores in a 7% polyacrylamide gel. Reduction of the disulfide bonds permits the electrophoretic resolution of *p*-κ-casein into two principal bands (Mackinlay and Wake, 1964; Kalan and Woychik, 1965), the significance of which is as yet unknown. Since it was possible that specific components were responsible for the two observed *p*-κ components, we investigated the *p*-κ derivatives obtained from the isolated major and minor components of B-CAM-κ-casein. Electrophoresis of *p*-κ derivatives of these components indicated approximately identical patterns, *i.e.*, in each case two bands were observed. Similar results were also obtained from the *p*-κ derivatives of A-CAM-κ-casein and its isolated major component. These results are shown in Figure 4. It therefore appears possible that both bands in *p*-κ arise as a direct result of rennin action on an individual component rather than to differences in the electrophoretic mobilities of *p*-κ-caseins formed from different components. The charged groups responsible for the differences in electrophoretic mobilities among the κ components are apparently associated with the MP portion of the molecule. As has already been suggested (Mackinlay and Wake, 1964; Woychik, 1965b), the minor gel electrophoresis bands of κ-casein may be attributable to charge differences arising from variations in carbohydrate content (sialic acid, hexosamine).

Sedimentation Studies of the Major κ-Casein Components. The high degree of association exhibited by κ-casein in neutral solutions has prevented the determination of the monomer molecular weight under normal conditions of pH and ionic strength. Determination of the monomer weight is further complicated by the presence of extensive intermolecular disulfide bonding which also results in a spectrum of molecular aggregates. In the presence of dissociating agents (urea, guanidine hydrochloride) the monomer molecular weight was reported to be of the order of 60,000 (Swaisgood and Brunner, 1963). This was substantially higher than the values of 16,000–26,000 (McKenzie and Wake, 1959; Waugh, 1958; Swaisgood and Brunner, 1962) obtained under alkaline conditions. This reduction in molecular weight can be attributed to cleavage of intermolecular disulfide bonds at high pH, presumably by β elimination. Molecular weights of the same order (18,000–20,000) were reported by Swaisgood and Brunner (1963) after reduction of κ-casein with 2-mercaptoethanol.

Sedimentation equilibrium experiments were done on the isolated major CAM-κ-casein components A-1 and B-1 at 25° in the presence of 5 M guanidine hydrochloride–(0.05 M potassium acetate–acetic acid (pH 5.3)). Molecular weights were determined according to the procedure of Yphantis (1964). The molecular weights found for components A-1 and B-1 were 17,800 and 18,400, respectively. The plots of the logarithm of fringe displacement against the square of the radial distance (Yphantis, 1964) were linear. This is indicative of homogeneity of molecular weight. The values thus obtained for the individual isolated components agree

TABLE I: Amino Acid Composition[a] of the Major Component of κ-Caseins A and B.

Amino Acid	κ-Casein Component A-1	95% Confidence Limits	Nearest Integer	κ-Casein Component B-1	95% Confidenc Limits	Nearest Integer
Aspartic acid	12.2	0.16	12	11.1	0.12	11
Threonine	14.3[b]		14	13.3[b]		13
Serine	12.5[b]		12–13	12.5[b]		12–13
Glutamic acid	27.0	0.33	27	26.8	0.25	27
Proline	19.6	0.27	20	20.0	0.21	20
Glycine	3.1	0.04	3	2.7	0.03	3
Alanine	13.5	0.18	13–14	14.3	0.15	14
Valine	10.5	0.15	10–11	10.9	0.13	11
Methionine	1.8	0.02	2	1.8	0.05	2
Isoleucine	11.2	0.14	11	12.2	0.12	12
Leucine	7.7	0.11	8	8.1	0.08	8
Tyrosine	7.8	0.13	8	7.9	0.08	8
Phenylalanine	4.1	0.05	4	4.1	0.05	4
Lysine	9.4	0.12	9	8.9	0.11	9
Histidine	3.2	0.05	3	3.3	0.04	3
Arginine	5.1	0.07	5	4.8	0.05	5
SCM-cysteine	1.9	0.03	2	1.8	0.02	2

[a] Values based on molar ratios (see text). [b] Values estimated using linear regression analysis.

well with the value reported in the literature for reduced whole κ-casein (Swaisgood and Brunner, 1963).

Amino Acid Composition of CAM-κ-Casein Components. Table I shows the amino acid composition of the reduced and alkylated major component of κ-caseins A and B, exclusive of tryptophan. Tryptophan values calculated for whole κ-casein (Kalan and Woychik, 1965) based on the reported extinction coefficient (Zittle and Custer, 1963) indicated a value of two residues of tryptophan per molecule of κ-casein (mol wt 19,000). It is assumed that the κ-casein components reported in this study also have a tryptophan content of two residues per molecule.

It is seen that the two major components have very similar compositions except for aspartic acid, threonine, alanine, and isoleucine. The component designated κ-casein A-1 has one residue more of aspartic acid and threonine than κ-casein B-1 which has one residue more of alanine and of isoleucine. All other amino acids differ by 0.5 residue or less. Summation of the residue weights (including two residues of tryptophan) for the major components A-1 and B-1 yielded values of approximately 19,000. These values agree well with the molecular weights calculated from the sedimentation equilibrium data.

The *S*-carboxymethylcysteine content of two residues per mole of κ-casein component is substantially higher than the value reported for κ-casein by Swaisgood and Brunner (1963), but comparable to that reported by Jollès *et al.* (1962).

Carbohydrate Analyses. Carbohydrate analyses of the major components A-1 and B-1 indicated the virtual absence of both hexose (galactose) and sialic acid.

Preliminary determinations on the minor components indicated the presence of carbohydrate (both sialic acid and hexose). The carbohydrate content appeared to increase in molar increments with the increased electrophoretic mobilities of the components. These preliminary results must be substantiated by a thorough carbohydrate analysis of the minor components; however, it appears that variations in carbohydrate are at least partly responsible for the electrophoretic differences of the minor κ-casein components.

Amino Acid Composition of Minor CAM-κ-Casein Components. Table II shows the amino acid compositions of two minor components (A-2 and A-4) of type A κ-casein and one minor component (B-2) of the type B κ-casein. The analyses of these components are somewhat less certain because of their relatively higher carbohydrate content and a lesser state of purity in comparison with the major κ-casein components. Nevertheless, the amino acid compositions of the minor components are remarkably similar to the major component with which they are associated. In the case of the minor component designated κ-casein B-2, the number of residues found for each amino acid is the same as the number found in the major B component, within 0.4 residue or less except for threonine and valine, where the difference is somewhat greater.

The minor components designated A-2 and A-4 contain a greater number of compositional differences between each other and between the major A compo-

TABLE II: Amino Acid Composition[a] of Some Minor Components of κ-Caseins A and B.

Amino Acid	κ-Casein A-2	95% Confidence Limits	Nearest Integer	κ-Casein A-4	95% Confidence Limits	Nearest Integer	κ-Casein B-2	95% Confidence Limits	Nearest Integer
Aspartic acid	12.0	0.16	12	12.0	0.20	12	11.1	0.21	11
Threonine	15.0[b]		15	14.8[b]		15	12.6[b]		13
Serine	13.8[b]		14	11.2[b]		11	12.2[b]		12
Glutamic acid	26.7	0.35	27	26.0	0.37	26	26.6	0.49	27
Proline	20.3	0.29	20	21.0	0.33	21	20.1	0.44	20
Glycine	3.7	0.05	4	3.1	0.09	3	2.4	0.06	2
Alanine	14.4	0.18	14	14.2	0.21	14	13.9	0.27	14
Valine	10.8	0.14	11	11.1	0.16	11	10.3	0.21	10
Methionine	1.9	0.03	2	2.0	0.03	2	1.8	0.03	2
Isoleucine	11.5	0.15	11–12	11.3	0.16	11	12.3	0.20	12
Leucine	8.2	0.11	8	8.5	0.13	8–9	7.8	0.14	8
Tyrosine	8.3[b]		8	8.1[b]		8	8.1[b]		8
Phenylalanine	3.8	0.05	4	3.6	0.05	4	4.1	0.09	4
Lysine	8.6	0.11	9	8.8	0.16	9	9.0	0.18	9
Histidine	3.3	0.05	3	3.1	0.05	3	3.4	0.08	3
Arginine	4.7	0.06	5	4.5	0.08	4–5	5.2	0.10	5
SCM-cysteine	1.3	0.02	1	1.4	0.03	1	1.8[b]		2

[a] Values based on molar ratios (see text). [b] Values estimated using linear regression analysis.

nent, A-1, with which they are associated. This is particularly true for the amino acids threonine, serine, glutamic acid, proline, and glycine. Nevertheless, these differences are not exceedingly large in terms of residue numbers. The low values for *S*-carboxymethylcysteine in the two minor A-κ components may be due to a greater destruction during acid hydrolysis, and it is therefore believed that the true value should be two residues per molecule of component.

Discussion

The ability to isolate the major and minor components from individual κ-casein preparations can be attributed to the reduction of the aggregates formed by intermolecular disulfide bonds. Once κ-casein has been reduced to monomeric units, normal chromatographic procedures can be applied to effect their separation and isolation. The imidazole–urea system used in this study has been used previously in casein chromatography (Ribadeau Dumas *et al.*, 1964; Rose and Marier, 1963), and with the reduction of κ-casein, it has permitted the isolation of the individual κ-casein components.

Although reduced and alkylated κ-casein has been used in this study, isolation of individual components from reduced nonalkylated κ-casein has been obtained with comparable results. However, in the case of nonalkylated κ-casein, the components again undergo aggregation due to intermolecular disulfide bonding, which might produce undesirable effects in further studies.

Recent evidence (Mackinlay and Wake, 1964; Woychik, 1965b) and that obtained in the present study seem to indicate that the basic unit of κ-casein is the individual electrophoretic component. The ability of the isolated major and minor components to stabilize α_{s1}-casein in the presence of calcium ions and their susceptibility to the action of rennin support this hypothesis. The formation of identical *p*-κ-casein derivatives from both the whole κ-casein and its isolated components suggests that the "*para* portion" of the molecules is identical in all of the components. Thus, it would appear that charges responsible for the different migration rates of the components reside in the portion of the polypeptide chain split off by the action of rennin. The presence of carbohydrate (galactose, sialic acid, and presumably galactosamine) in the minor components and its absence in the major components suggest that the carbohydrate content contributes to this charge distribution. Differences in amino acid compositions may also be factors in the heterogeneity within individual κ-caseins.

There is no independent evidence at the present time to ascertain how the aspartic acid–threonine and alanine–isoleucine differences between the major A-1 and B-1 components are paired. That the amino acid differences are paired is inferred from the presumed genetic polymorphism of the proteins. In addition, it was found in the case of the whey protein, β-lactoglobulin, that β-lactoglobulin A differs from β-lactoglobulin B in having one residue fewer of glycine and alanine and one residue more of aspartic acid and valine (Gordon *et al.*, 1961; Piez *et al.*, 1961). In this instance, it has been

shown that the amino acid differences are paired as substitutions Asp/Gly and Val/Ala (Kalan *et al.*, 1962). On the basis of the ribonucleic acid (RNA) code as reported by Nirenberg *et al.* (1965), both Thr/Ile and Thr/Ala substitutions are possible and these single amino acid substitutions have been reported in other proteins as summarized by Jukes (1965). An Asp/Ile substitution has not, as far as we know, been reported in proteins and seems unlikely on the basis of the RNA code. However, there has been a report of an Asp/Ala substitution in tobacco mosaic virus (Wittman and Wittmann-Liebond, 1963) and this substitution is more likely than Asp/Ile on the basis of the RNA code. Therefore, it is concluded that the amino acid differences found by analysis are most likely related by the substitutions Asp/Ala and Thr/Ile. The extra aspartic acid residue in κ-casein component A-1 probably accounts for its greater mobility in gel electrophoresis compared with component B-1. In the above discussion it has been assumed that the amino acid differences involve aspartic acid and not asparagine.

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