

PURIFICATION AND SOME OF THE PROPERTIES OF α_s -CASEIN AND κ -CASEIN

SUMMARY

α_s -Casein and κ -casein have been freed of major contaminants, judged by starch-gel-urea electrophoresis. The α_s -casein was prepared by a urea-sodium chloride procedure with a final precipitation of impurities by ethanol. κ -Casein was prepared by extraction of whole casein with urea-sulfuric acid supplemented with ethanol precipitation. Electrophoretic mobility, pH of minimal solubility salt-free (isoelectric point), and with 0.1 M NaCl (isoelectric point), ultraviolet absorption, and interaction with calcium ions are reported.

Starch-gel-urea electrophoresis has provided a new and more effective means for estimating the purity of the caseins (10). With this procedure as a guide, new and relatively simple methods have been devised for the purification of κ -casein and α_s -casein. κ -Casein was purified by extraction of whole casein with urea-sulfuric acid (13) supplemented with ethanol precipitation (6). The α_s -casein was purified by a urea-sodium chloride procedure (15) with a final ethanol precipitation. Details of the purification methods are described herein, together with some of the properties of these purified caseins [electrophoretic mobility, pH of minimal solubility salt-free (isoelectric point), and with 0.1 M NaCl (isoelectric point), ultraviolet absorption, and interaction with calcium ions].

MATERIALS AND METHODS

Whole casein was prepared from pooled milk by precipitation with HCl at pH 4.7. The casein was drained and squeezed in a cloth bag to reduce the water content and stored at -20°C .

Starch-gel electrophoresis was performed in 7 M urea at pH 8.6 as described by Wake and Baldwin (10).

Free-flow electrophoresis by the Tiselius technique was done with Perkin-Elmer equipment, Model 38.² Buffers, etc., used are referred to later.

Details of the other methods used are described under Experimental.

Received for publication July 18, 1963.

¹ Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

² It is not implied the U. S. Department of Agriculture recommends the above company or its products to the possible exclusion of others in the same business.

EXPERIMENTAL PROCEDURES AND RESULTS

Preparation of κ -casein. The preparation method has been reported in abstract previously (13). A frozen block of acid-precipitated whole casein weighing about 350 g (60 to 95 g of protein) is dissolved in one liter of 6.6 M urea. This solution is acidified with 200 ml of 7 N H_2SO_4 (one part concentrated to four parts water). After acidification two liters of water are added. The pH of the mixture is 1.3 to 1.5. No precipitate is apparent at first, but the turbidity gradually increases and a flocculent precipitate forms. After standing for 2 hr the precipitate is filtered off and discarded or can be used for the preparation of α_s - and β -caseins. The κ -casein in the filtrate is precipitated by the addition of 132 g (1 M) ammonium sulfate to each liter of filtrate. The precipitate is collected, suspended in water, and dissolved by the addition of 1 N NaOH to a final pH of 7.5. The solution is dialyzed and freeze-dried. The yields of κ -casein have been 7 to 12% of the whole casein used. Preparations dried at a lower pH are usually very slow to dissolve, even with the addition of NaOH to raise the pH to 7.5. Such preparations do, however, eventually dissolve with apparently no loss in their stabilizing power. Some preparations gave solutions that were slightly turbid, presumably due to traces of lipide. Extraction of the dry preparations with organic solvents (acetone, ethanol, ether) to remove the lipides impaired the solubility of the κ -casein and its ability to stabilize the α_s -casein against precipitation with CaCl_2 . This loss of stabilizing power did not occur with less pure κ -casein preparations when treated with lipide solvents.

This procedure often gives products with only a small amount of α_s -casein as a contaminant (Figure 1, center). Occasionally the

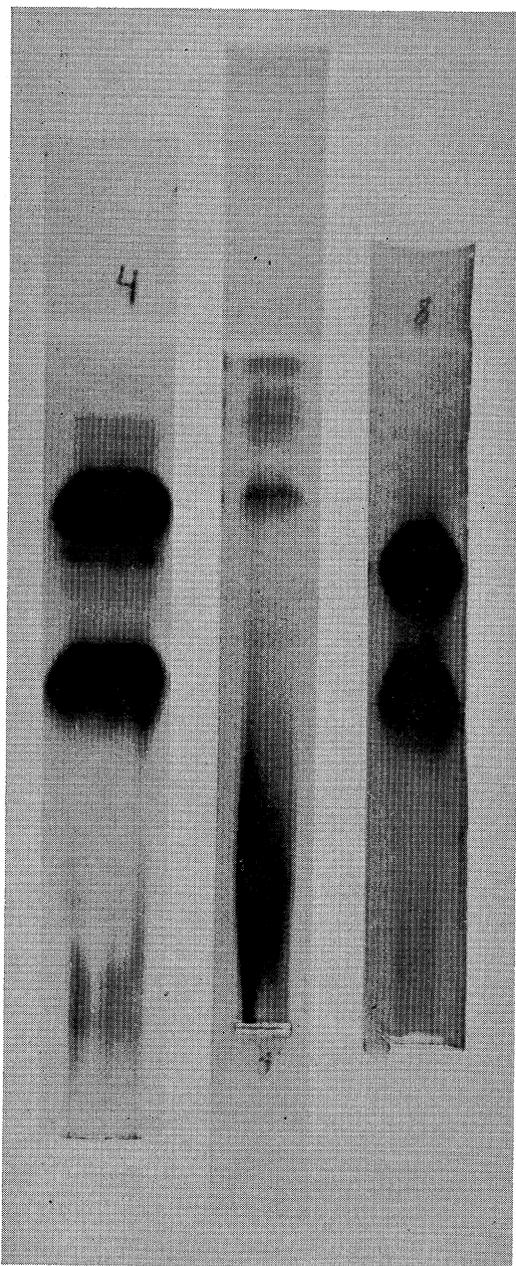


FIG. 1. Starch-gel-urea electrophoresis diagrams of κ -casein fractionation by sulfuric acid method. Point of application at bottom. Left: Whole casein. Center: κ -Casein preparation containing α_s -casein as major contaminant. Right: Portion of whole casein insoluble in sulfuric acid.

κ -casein preparations have contained larger amounts of protein contamination (Figure 2, left). These preparations have been freed of contaminants by ethanol precipitation (6). This was done as follows: One volume of a 1% pH

7.0 solution of κ -casein prepared by the sulfuric acid method was mixed with two volumes

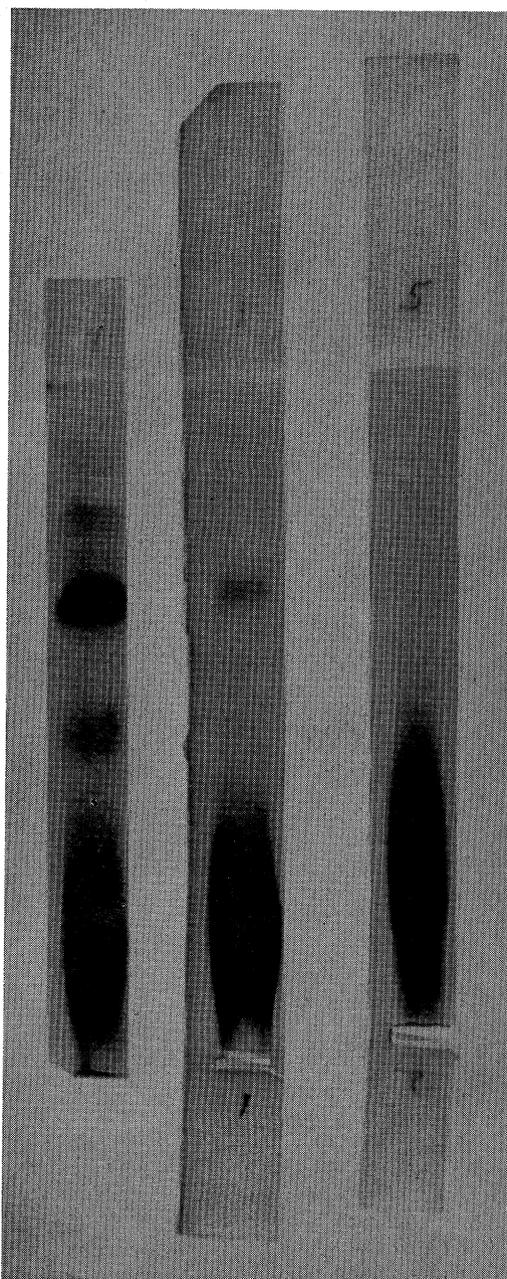


FIG. 2. Starch-gel-urea electrophoresis diagrams of κ -casein by the sulfuric acid method after further purification with ethanol. Point of application at bottom. Lower diffuse band in each diagram is κ -casein; upper heavy band is α_s -casein. Left: Preparation containing both α_s -casein and β -casein. Center: Preparation shown at left after one precipitation with ethanol. Right: Preparation after second precipitation with ethanol.

of ethanol. One molal ammonium acetate in 75% ethanol was added until the typical sticky precipitate was obtained. The precipitate was dissolved in water with addition of NaOH to bring the pH to 7.5, dialyzed, and freeze-dried. Results of two precipitations by this method are shown in Figure 2.

Preparation of α_s -casein. α_s -Casein was prepared by a modified urea procedure, in which extra NaCl was added to dissociate the α -casein complex (15). The preparation appeared to be homogeneous on free-flow electrophoresis at pH 2.3 (15), but starch-gel electrophoresis in urea showed that there was considerable contamination with a number of contaminants, including β -casein, a band just ahead of β -casein and κ -casein (Figure 3a). These contaminants were almost completely removed by precipitation with ethanol. A 2% aqueous solution of the α_s -casein was prepared at pH 7.2. One volume of this solution was mixed with one

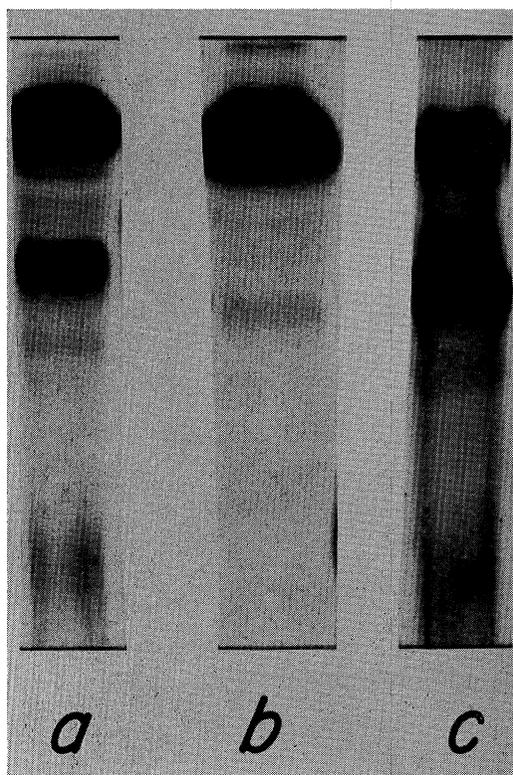


FIG. 3. Starch-gel-urea electrophoresis diagrams of α_s -casein (urea-NaCl method) before (a) and after (b) purification with ethanol. Point of application at bottom. (a) α_s -Casein (top heavy band) showing contamination with both β -casein (narrow heavy band) and κ -casein (bottom diffuse band). (b) α_s -Casein after ethanol purification. (c) Components of ethanol precipitate.

volume of ethanol, and 1 M ammonium acetate in 75% ethanol was added (about 0.06 volume) until maximum precipitation was obtained. This precipitate contained the contaminants (Figure 3c) and components that contributed turbidity to the α_s -casein solutions. The α_s -casein in the ethanol was recovered by acidification with 3 N HCl (about 0.02 volume) to a pH of 5.0. The α_s -casein precipitate was recovered, dissolved at pH 7.5 with the addition of NaOH, dialyzed, and freeze-dried. The α_s -casein recovered was about 75% of the starting material, the impure α_s -casein (15). Starch-gel electrophoresis of this product is shown in Figure 3b. The purified α_s -caseins are readily soluble and give water-clear solutions.

Properties of the casein. Some properties of κ -casein and α_s -casein obtained by the above methods are given in Table 1.

Solutions of the κ -casein and the α_s -casein were desalted by passing them through a column containing a mixed bed resin, Amberlite MB-1,² a mixture of the anion exchange resin IRA-400 and the cation exchange resin IR-120. The column of resin was 18 by 3 cm. The column was washed several times with water. Approximately 500 mg of the casein in 25 ml of water at pH 7.0 was placed on the column and washed through with water. The absorbent was not completely inert to the casein, for only 60 to 70% of the casein was washed through with water. (The remainder of the casein could be eluted with NaOH or NaCl. These fractions did not appear to differ significantly from the water fraction.) The water fraction was strongly opalescent and was at a pH of 5.3 for α_s -casein, 5.4 for κ -casein. Dilution of these solutions to 0.1% concentration and addition of minute amounts of 0.02 N HCl gave flocculent precipitates and the pH values indicated in Table 1 for the isoionic points. With 0.1 M NaCl added to these solutions the pH zone of flocculation was much broader, but the midpoint pH values for the isoelectric points are given in Table 1.

Approximate values for the isoionic points were also obtained from titrations of these solutions with and without 0.1 M NaCl present. With α_s -casein the salt curve crossed the non-salt curve at pH 4.9, whereas with κ -casein the curves crossed at pH 5.4. The large shift in the isoelectric point of κ -casein with NaCl is supported by the marked shift in the pH of solutions at a pH above the isoionic point on the addition of NaCl to still higher pH values, suggesting that chloride ion was bound and thereby accounting for the marked drop in the isoelectric point with added salt. (In general,

TABLE 1
Properties of κ -casein and α_s -casein

Preparation	Phosphorus ^b	Nitrogen ^b	Ash ^c	Sialic acid ^{b, d}	Extinction 0.1%, 278 $m\mu$ ^e	Isoelectric point ^f	Isoionic point ^g
κ -casein	0.30	15.4	1.6	1.94	1.22	3.7	5.1
α_s -casein ^a	1.01	15.1	5.4	0.08	1.02	4.4	4.7

^a The α_s -casein was prepared from commercial pooled milk. It was, however, the genetic variant B (9a) on the basis of starch-gel electrophoresis and amino acid analysis (the amino acid analyses were provided by Dr. William G. Gordon).

^b Calculated on moisture-free basis.

^c Fixed ash; magnesium acetate added.

^d We are indebted to Dr. Dyson Rose, National Research Council, Ottawa, Canada, for these analyses. They were done by the recently developed method of Marier, Tessier, and Rose (5a).

^e Extinction in 1-cm cell, pH 7.5. Extinction values at 275 $m\mu$ and 299.4 $m\mu$ in 0.1 M NaOH were used to calculate the tyrosine and tryptophan content (2). κ -Casein gave values of 8.0 and 2.1%, respectively, α_s -casein values of 8.8 and 2.3, close to values obtained by other means [Dr. William G. Gordon (2a); unpublished results].

^f Values obtained by extrapolation of free-flow electrophoretic results in 0.1 ionic strength buffers and pH of minimal solubility in 0.1 M NaCl.

^g pH of minimal solubility in solutions freed of salts by passage through a mixed-bed resin column.

nonspecific effects of added salts lead to pH increases below the isoionic point and pH decreases above the isoionic point.) Free-flow electrophoresis, however, with several concentrations of NaCl (constant buffer) did not give any mobility shift that could be attributed to chloride binding.

Monovalent buffer-NaCl mixtures (7) and NaCl-HCl mixtures (pH 2 and less) were used for the mobility determinations at an ionic strength of 0.1. For the few measurements at ionic strength 0.05 the buffer concentration remained the same, but the NaCl was decreased by 0.05 M. The isoelectric pH range of low solubility was broad in the presence of salts and the caseins were poorly soluble. In this range the caseins were dissolved in the buffer and the required amount of solid NaCl added. In most experiments the concentration of casein was 1%, but bordering the isoelectric range the concentration was about one-half as much. The results are shown in Fig. 4.

The κ -casein is readily clotted by rennin, but the nature of the clot is determined by the salts present. Ten-milliliter portions of 0.25% κ -casein, adjusted to a final pH of 6.2 after the addition of salts, were tested with commercial rennin (0.4 mg) in the following systems: (1) No salts, (2) 0.1 M NaCl, (3) 0.1 M Na phosphate, (4) 0.010 M CaCl₂, (5) 0.01 M CaCl₂ with 0.002 M Na phosphate. Number 4 flocculated in 5 min, numbers 2, 3, and 5 became white within 5 min and formed cohesive, contracting clots within 20 min, and number 1 became opalescent within 5 min and flocculated within 20 min.

DISCUSSION

κ -Casein is clotted by rennin without calcium ions (8, 11) and it is readily clotted in the presence of 0.1 M NaCl (6). The present studies show that salts are required for the rapid formation of a good clot. The requirement appears to be nonspecific, acting perhaps by repressing ionic repulsion, since both sodium chloride and sodium phosphate have similar effects. With calcium ions (0.010 M) a small amount of phosphate (0.002 M) promotes clot formation as is the case with the whole α -casein complex (14). Without any added ions (system No. 1) a clot is not formed and flocculation is slower but to the same degree (measured by ultraviolet absorption at 280 $m\mu$) as with salts present. Whether this slow flocculation represents a change (proteolysis) beyond that required for specific clotting needs further study.

The extraction of κ -casein from acid-precipitated casein with dilute sulfuric acid provides a simple method for preparing this casein in relatively pure form with the retention of its unique stabilizing property, sialic acid, etc. Furthermore, the insoluble portion, principally α_s - and β -caseins, can serve for the isolation of these caseins. The κ -casein prepared by this method stabilizes α_s -casein against precipitation with calcium chloride, as described previously in detail (14). κ -Casein kept 80% of α_s -casein in solution with 0.01 M CaCl₂ at a κ/α_s ratio of 1:10. Ethanol precipitation, although effective in completely purifying the κ -casein, did not lead to an increase in the sta-

bilizing power of the κ -casein. In fact, some preparations were less effective as stabilizers, which suggests that the ethanol precipitation be used with caution and that the stabilizing power of the κ -casein be tested after such treatment. Recently, κ -casein has been reported (1, 3) to dissociate in urea to give a product resembling para- κ -casein. This is certainly not true of TCA-urea preparations of κ -casein (9) or the present H_2SO_4 -urea preparations, for the solubility of the products is good and the κ -casein by both methods retains its unique ability to stabilize α_s -casein against precipitation by $CaCl_2$. On the other hand, the ethanol precipitation may bring about a dissociation of the type described (1, 3), in view of the detrimental effect noted occasionally. Alteration of tertiary structure (denaturation) is another possibility in view of the action of lipide solvents on the dry κ -casein.

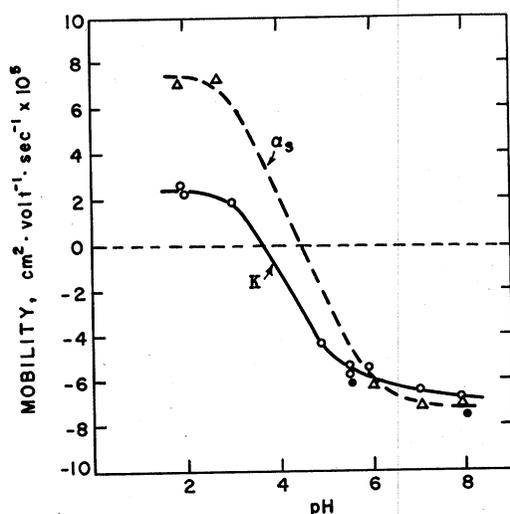


FIG. 4. Mobility of α_s -casein and κ -casein obtained by free-flow electrophoresis at various pH values. Conditions of electrophoresis are described in text. α_s -Casein; ionic strength 0.1— Δ . κ -Casein, ionic strength 0.1— \circ . κ -Casein, ionic strength 0.05— \bullet .

The use of ethanol in the final purification of α_s -casein was very effective in purifying this casein and in removing the components responsible for turbid solutions. The preparations were readily soluble.

The desalting of κ - and α_s -casein solutions with the mixed-bed resin gave solutions suitable for determining the salt-free isoelectric point, the so-called isoionic point. The values found, 5.1 and 4.7, for κ - and α_s -caseins, respectively, are in the order expected from the composition of these caseins (4, unpublished

studies): That is, κ -casein is the more basic protein. (κ -Casein is quite comparable to α_s -casein in amino acid composition, although they differ in solubility, stabilizing power, etc.) The isoelectric points of κ -casein and α_s -casein in the presence of salts, from both free-flow electrophoresis and pH of minimum solubility, are in the reverse order, namely 3.7 and 4.4, respectively, suggesting that α_s -casein is the more basic protein. This presumably comes about through a binding of anions to the κ -casein. Hipp et al. (5) concluded from free-flow electrophoretic curves similar to those obtained in the present study that α_s -casein had a lower isoelectric point than α_1 -casein. Swaisgood and Brunner (9) obtained an isoelectric point for κ -casein of 3.8 from free-flow electrophoresis, and 3.8-4.2 from minimum solubility in the presence of salts.

REFERENCES

- (1) BEEBY, R., AND NITSCHMANN, H. The Action of Rennin on Casein. The Disruption of the κ -Casein Complex. *J. Dairy Research*, 30: 7. 1963.
- (2) GOODWIN, T. W., AND MORTON, R. A. The Spectrophotometric Determination of Tyrosine and Tryptophan in Proteins. *Biochem. J.*, 40: 628. 1946.
- (2a) GORDON, W. G., AND BASCH, J. J. Tryptophan Content of Purified Milk Proteins. *Biochim. et Biophys. Acta*, 48: 397. 1961.
- (3) HILL, R. D. The Preparation of κ -Casein. *J. Dairy Research*, 30: 101. 1963.
- (4) HIPPI, N. J., BASCH, J. J., AND GORDON, W. G. Amino Acid Composition of α_1 , α_2 , and α_s -Caseins. *Arch. Biochem. Biophys.*, 94: 35. 1961.
- (5) HIPPI, N. J., GROVES, M. L., AND McMEEKIN, T. L. Separation of the Components of α -Casein. II. The Preparation of α_s -Casein. *Arch. Biochem. Biophys.*, 93: 245. 1961.
- (5a) MARIER, J. R., TESSIER, H., AND ROSE, D. Sialic Acid as an Index of the κ -Casein Content of Bovine Skimmilk. *J. Dairy Sci.*, 46: 373. 1963.
- (6) MCKENZIE, H. A., AND WAKE, R. G. An Improved Method for the Isolation of κ -Casein. *Biochim. et Biophys. Acta*, 47: 240. 1961.
- (7) MILLER, G. L., AND GOLDBER, R. H. Buffers of pH 2 to 12 for Use in Electrophoresis. *Arch. Biochem.*, 29: 420. 1950.
- (8) NITSCHMANN, H., AND BEEBY, R. Das Lab und Seine Wirkung auf das Casein der Milch. XIV. Aminosäure-Zusammensetzung des aus κ -Casein durch Lab in Freiheit Gesetzten Glyko-Makropeptids. *Chimia*, 14: 318. 1960.
- (9) SWAISGOOD, H. E., AND BRUNNER, J. R. Characterization of κ -Casein Obtained by Fractionation with Trichloroacetic Acid in

- a Concentrated Urea Solution. *J. Dairy Sci.*, 45:1. 1962.
- (9a) THOMPSON, M. P., KIDDY, C. A., PEPPER, L., AND ZITTLE, C. A. Variations in the α_s -Casein Fraction of Individual Cow's Milk. *Nature*, 195:1001. 1962.
- (10) WAKE, R. G., AND BALDWIN, R. L. Analysis of Casein Fractions by Zone Electrophoresis in Concentrated Urea. *Biochim. et Biophys. Acta*, 47:225. 1961.
- (11) WAUGH, D. F., AND VON HIPPEL, P. H. κ -Casein and the Stabilization of Casein Micelles. *J. Am. Chem. Soc.*, 78:4576. 1956.
- (12) ZITTLE, C. A. Stabilization of Calcium-Sensitive (α_s) Casein by κ -Casein: Effect of Chymotrypsin and Heat on κ -Casein. *J. Dairy Sci.*, 44:2101. 1961.
- (13) ZITTLE, C. A. Procedure for Isolation of κ -Casein by Use of Sulfuric Acid. *J. Dairy Sci.*, 45:650. 1962.
- (14) ZITTLE, C. A., AND CERBULIS, J. Clotting of Casein with Pepsin: Amount and Nature of the Soluble Products. *J. Dairy Sci.*, 41:241. 1958.
- (15) ZITTLE, C. A., CERBULIS, J., PEPPER, L., AND DELLAMONICA, E. S. Preparation of Calcium-Sensitive α -Casein. *J. Dairy Sci.*, 42:1897. 1959.