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The α_s-Caseins of Bovine Milk[1]

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Fractionation of crude α-casein, obtained by calcium precipitation, has been performed on DEAE-cellulose at *p*H 7, 13° in the presence of 4.5 *M* urea, using stepwise increments in ionic strength to elute. Two major components, accounting for 45% of the optical density of first cycle casein, have the same adsorption characteristics and are eluted together at ionic strength 0.18. These are α_s-caseins, defined here as those caseins which interact with κ-casein at appropriate weight ratios, forming complexes in the absence of divalent cations and micelles clottable with rennin in the presence of divalent cations. Starch gel electrophoresis in the presence of 6.5 *M* urea, to date the only method to reveal the presence of two materials, resolves them at *p*Hs 3.2, 4.3 and 8.4 into two sharp bands having nearly the same mobility. The component of greater mobility at *p*H 8.4 is termed α_{s1}-casein and the other α_{s2}-casein. The average molecular weight of $\alpha_{s1,2}$-caseins by measurement of tryptophan released by carboxypeptidase A is 27,000 to 27,500 and by osmotic pressure measurements in 6.5 *M* urea at *p*H 4.5 is 26,900 ± 2000. The correspondence of these and the light scattering value at *p*H 12 of 27,300 ± 1500, reported elsewhere, establishes monomer size. $\alpha_{s1,2}$Caseins contain 14.7% N, 1.03% P, 2.3 tryptophans and 11 tyrosines per 27,300 g., and are free of carbohydrate, cystine and cysteine. The $E_{1\%}$ at λ = 2800 Å. is 10.1. Carboxypeptidase A can release one mole each of tryptophan, tyrosine and leucine from 27,300 g. of protein; basic or acidic C-terminal amino acids were not found. C-terminal studies of cleavage products establish tryptophan and leucine and suggest tyrosine as terminal carboxyl groups. The $\alpha_{s1,2}$-caseins are evidently similar components. α_s-Casein fractions, eluted from DEAE-cellulose columns after $\alpha_{s1,2}$-caseins, are paucidisperse by starch gel analysis, yield mainly C-terminal tyrosine and contain 1.15 to 1.35% P, more P than $\alpha_{s1,2}$-, β- or κ-caseins.

Casein micelles, the colloidal particles of milk, result from a set of specific component interactions and constitute a system in true and rapid equilibrium with components in solution.[3] Almost all of the interactants are macromolecules. An enzymatic alteration in one of the less abundant components, κ-casein, is sufficient to produce the phenomena associated with clotting.[4]

The total number of casein components present in milk is not known, but this number is certainly above 8 and has been set as high as 20 by Wake and Baldwin.[5] Eventually it will be necessary that we know which of these components are produced by the synthetic mechanisms of the cells (primary caseins) and which are derived from primary caseins by subsequent proteolysis. At this time it seems reasonable to assume that at least the four most abundant caseins ($\alpha_{s1,2}$-caseins, β-casein and κ-casein) are primary caseins. Fractionation and characterization of these has been set as a necessary step toward studies of casein interactions.

One of the major problems in fractionation and characterization has been the fact that several of the caseins interact strongly with each other, either in the presence or in the absence of calcium. In 1957 we found that concentrations of urea above 4.2 *M* will effectively dissociate interaction products into monomers. Since then a fractionation procedure employing DEAE-cellulose in the presence of urea has been developed to separate individual components.

Our purpose here is to discuss the α_s-caseins. The designation α_s has been chosen for two reasons: first, to group together components which have the property of engaging in interaction with κ-casein in micelle formation and in formation of the clot structure (3, 6, see Discussion); and second, to retain a relationship with other α-casein preparations, such as that of Warner,[7] which contain α_s-caseins as major portions. If there were only one α_s-casein the problem of naming would be simple. Evidence will be presented here to show that there are two and possibly several α_s-casein components.

Two α_s-caseins, termed α_{s1}- and α_{s2}-caseins, account for at least 45% of the total casein optical density. Because their adsorption–elution characteristics on DEAE-cellulose are so nearly the same they are isolated together and will be referred to as $\alpha_{s1,2}$-caseins. α_{s1}- and α_{s2}-caseins appear to have similar properties. Indeed, the only method which has so far revealed the presence of two components is starch gel electrophoresis. Other proteins obtained by the fractionation procedure have not been adequately characterized and will be referred to as α_s-casein fractions.

This publication deals with the fractionation of the α_s-caseins and particularly with certain physical and chemical properties of $\alpha_{s1,2}$-caseins.

Materials and Methods

Chemicals.—All were Analytical Reagent Grade. Stock solutions of calcium chloride, 5 *M*, and potassium citrate, 1 *M*, were clarified of suspended material by centrifugation. Except for urea, other reagents were used without special treatment.

Distilled Water.—The general supply of distilled water was passed through a Barnstead mixed bed resin cartridge and then through an HA Millipore filter. The pH was between 5.5 and 7.0.

Urea and Urea Buffers.—Analytical Reagent Grade urea supplied by Mallinckrodt Chemical Co. was used. This urea was found to contain dark colored colloidal materials, which were removed by DEAE-cellulose columns, and heavy metal ions, which could be accumulated by the caseins during preparative procedures. In addition, commercial urea was found to contain cyanate, which may react with lysyl

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(3) D. F. Waugh, *J. Phys. Chem.*, **65**, 1793 (1961).

(4) D. F. Waugh, *Discussions Faraday Soc.*, **25**, 186 (1958).

(5) R. G. Wake and R. W. Baldwin, *Biochem. et Biophys. Acta*, **47**, 225 (1961).

(6) D. F. Waugh and P. H. von Hippel, *J. Am. Chem. Soc.*, **78**, 4576 (1956).

(7) R. C. Warner, *ibid.*, **66**, 1725 (1944).

groups.[8] A satisfactory solution was obtained as follows. A 25 lb. drum of urea was dissolved in 33 liters of distilled water and the solution passed through a thin layer of Johns-Manville "Celite" and then through IRC-50 in the sodium form. Next, 200 ml. of 2 *M* HCl were added to give a pH of 3.5. After standing at room temperature for 48 hours specific tests for cyanate according to the method of Werner[9] were negative. To the urea solution was then added sufficient imidazole to give a 0.01 M solution and the pH was adjusted to 7 with molar acid or alkali. Solutions containing 4.5 *M* urea and 0.01 *M* imidazole (pH 7) will be referred to as Buffer I. Additions of sodium chloride to Buffer I will be designated according to the added ionic strength; thus, B-I 0.15 is Buffer I containing 0.15 *M* sodium chloride. When not used immediately Buffer I was stored at 4° although the return of appreciable amounts of cyanate at room temperature and pH 7 was found to take several days.

Carboxypeptidase A.—Worthington[10] crystalline suspensions which had been treated with diisopropylfluorophosphate (DFP) were used. In some cases dilute solutions were prepared from the suspensions; more often, the amount of enzyme required for a single experiment was removed and dissolved by addition of buffer just prior to the experiment. The protein concentration of the suspensions was taken to be 5%.

Carboxypeptidase B.—Three preparations were used. One was Worthington CPB, some of which was made 0.01 *M* in DFP. The other preparations were made in the laboratory from commercial trypsin 1:300 following the procedure of Folk, *et al.*[11] By single assays using hippurylarginine as described by Folk, *et al.*, the activities were found to be: Worthington CPB, 10,000 units per mg. after DFP treatment; our preparations, 11,000 and 13,000 units per mg.

Trypsin.—Twice crystallized salt free trypsin was obtained from Worthington. Stock 1% solutions in 0.001 *N* HCl were stored in a freezer.

Identification of Amino Acids.—Appropriate aliquots of CPA digestion mixtures, containing 0.01–0.1 mole of amino acid, were streaked on Whatman 3MM paper. Drying occurred in a few minutes and was assumed to stop the reaction. The samples were subjected to high voltage electrophoresis (3.5 to 5 kv.) at pH 1.9 in 8.7% acetic acid, 2.5% formic acid[12,13] for times adequate to move lysine 64–76 cm. Final identification was obtained by two-dimensional separations in which 2-butanol: 3% ammonia (5:2) was used as a descending solvent after pH 1.9 electrophoresis. The presence of leucine or isoleucine was confirmed by adding leucine to one aliquot of the unknown, and isoleucine to another.

Quantitative Ninhydrin Determinations.—For estimates of the amounts of amino acids present after one or two dimensional separations, the procedure of Kay, Harris, and Entenman[14] was followed, except that the ninhydrin was buffered at pH 7–7.2. A standard amino acid mixture was always run in parallel with the unknowns to determine color yields for the particular amino acids.

Nitrogen.—Kjeldahl digestions and subsequent titrations were performed in this laboratory and by Dr. S. M. Nagy, Department of Chemistry, Massachusetts Institute of Technology, Cambridge.

Phosphorus.—The method of Bartlett[15] was used.

Carbohydrate.—The resorcinol method of Svennerholm[16] for sialic acid and the orcinol method of Schultze, *et al.*, for hexose[17] were used.

Osmotic Pressure.—Osmotic pressure determinations were carried out in the presence of 6.5 *M* urea, 0.1 *M* KCl at 25° using the modified Hepp osmometer as described by Scatchard, *et al.*[18] Professor George Scatchard, M.I.T., kindly loaned us an osmometer for these measurements. Schleicher and Schuell UA Very Dense membranes were conditioned by several days' soaking, first in water and then in 6.5 *M* urea, 0.1 *M* KCl solutions. Stock solutions of protein in urea were prepared by the addition of solid urea, solid KCl, and water to protein solutions which had been dialyzed against 0.1 *M* KCl. The pH was adjusted with HCl before final dilution. Measurements were made at a series of concentrations obtained by diluting stock protein solutions with 6.5 *M* urea, 0.1 *M* KCl adjusted to the required pH. After determination of the osmotic pressure the protein solutions were recovered from the cell. The pH was measured, and protein concentrations were determined from measurements of optical density at 280 mμ after dilution with ammonium bicarbonate buffer.

Starch Gel Electrophoresis.—The technique followed that described by Smithies.[19] The standard acetic acid gels contained 6 to 7 *M* urea and were prepared as follows: 58.5 g. of starch[20] was suspended in 95 ml. of distilled water, poured into 350 ml. of 9 *M* urea and heated with mechanical stirring to 70° when 34 ml. of glacial acetic acid were added. Stirring was continued briefly after which the mixture was degassed and poured. Bridge solutions contained 6.5 *M* urea, 7.5% acetic acid and electrode solutions were 5% sodium sulfate. The proteins were dissolved in 6.5 *M* urea to which was added a tenth volume of glacial acetic acid. Horizontal runs were made successfully by dissolving the samples in starch gel which had been diluted 1:1 with gel buffer while still warm, producing a viscous liquid which prevented convection in the slots. Gel pH values were near pH 3.3 as a result of the ammonia content of the urea. High current densities were achieved with the standard plastic tray of Otto Hiller[20] by cutting away most of the plastic backplate and replacing it with a water cooled brass plate covered with a film of vinyl butyrate about 0.010 inch (0.254 mm.) thick.

Tris-citrate gels of the type described by Wake and Baldwin[5] have been used according to the procedure given by those authors.

Several two dimensional starch gel electrophoresis runs were required. For these experiments the cooled starch tray was modified so that, after the first dimension the side pieces of the gel tray could be removed giving access to small troughs through which buffer bridges could carry current to the sides of the original gel. All unwanted parts of the initial gel were removed and plastic pieces inserted to define the new gel sides.

Preparative Centrifugation.—A Spinco Model L centrifuge and an International Model PR-2 have been used. Effective temperature control of the Model L rotor during centrifugation was achieved by inserting an air-sensitive thermistor through the lid.[21] The thermistor, in conjunction with a Yellowsprings Model 71 Thermistemp control, operated on the refrigeration unit. To maintain temperature the refrigeration unit was increased to 1/3 HP and Freon 22 was used as a refrigerant.

Dialysis Tubing.—Usually 18/32 Visking tubing was used, and all tubing was either leached at room temperature in 3% sodium carbonate for 24 hours or boiled for 10 min. in 3% sodium carbonate. Tubing was then rinsed in distilled water.

Diethylaminoethyl-cellulose.—DEAE-cellulose prepared by Brown Paper Co.[22] was used. Fine particles were first removed by suspending the cellulose three times in distilled water. It was then formed into columns which were washed alternately with B—I 0.5 containing 0.1 *M* NaOH and with B—I 0.5 containing 0.1 *M* HCl. For a column 8.4 cm. in diameter and 5 cm. long three washing cycles of one liter each of the acid and alkaline urea solutions were used. These were followed by 600 ml. of B—I 0.5 containing 0.15 *M*

(8) G. R. Stark, W. H. Stein and S. Moore, *J. Biol. Chem.*, **235**, 3177 (1960).

(9) E. A. Werner, *J. Chem. Soc.*, **123**, 2577 (1923).

(10) Worthington Biochemical Corporation, Freehold, New Jersey.

(11) J. E. Folk, K. A. Piez, W. R. Carroll and J. A. Gladner, *J. Biol. Chem.*, **235**, 2272 (1960).

(12) G. N. Atfield and C. J. O. R. Morris, *Biochem. J.*, **81**, 606 (1961).

(13) A. M. Katz, W. J. Dreyer and C. B. Anfinsen, *J. Biol. Chem.*, **234**, 2897 (1959).

(14) R. E. Kay, D. C. Harris and C. Entenman, *Arch. Biochem. Biophys.*, **63**, 14 (1956).

(15) G. R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).

(16) L. Svennerholm, *Biochem. et. Biophys. Acta*, **24**, 604 (1957).

(17) H. E. Schultze, R. Schmidtberger and H. Haupt, *Biochem. Z.*, **329**, 490 (1958).

(18) G. Scatchard, A. Gee and J. Weeks, *J. Phys. Chem.*, **58**, 783 (1954).

(19) O. Smithies, "Advances in Protein Chemistry," Vol. XIV, C. B. Anfinsen, Jr., K. Bailey, M. L. Anson and J. T. Edsall, editors, Academic Press, Inc., New York, N. Y., 1959, p. 65.

(20) Obtained from Otto Hiller, Toronto, Ontario, Canada.

(21) Yellowsprings Instrument Co., Yellow Springs, Ohio.

(22) Brown Paper Co., Berlin, New Hampshire. Reagent Grade Lot No. 1054. Capacity 1.0 meq. per gram.

imidazole adjusted to pH 7.0. Finally the column was washed with B-I.

Spectrophotometry.—Outputs of columns have been monitored with a Cary Model 11 recording spectrophotometer set at $\lambda = 2800$ Å.

Optical Density Unit.—A unit was defined as equal to the amount of protein which, when dissolved in 1 ml., had unit optical density in a 1 cm. cell at 2800 Å., pH 7.0.

Milk.—Milk was obtained at the time of milking and cooled at once. It was skimmed as soon as possible and stored at −15°.

First Cycle Casein.—The starting material for fractionation was first cycle casein, FCC. A preparative procedure for FCC has been described previously.[6,23] Some changes which simplify the procedure are given here.

The caseins were driven into micelle form by slowly adding to skim milk at pH 7 and 37° sufficient calcium chloride to make the milk 0.07 *M* in added calcium ion. Constancy of pH was maintained by adding 2 *M* tris (pH 10.2). Micelles were removed by centrifugation at 37° for 90 minutes in a Spinco Model L Centrifuge using the No. 20 rotor at 18,000 r.p.m.

Supernatants and residual cream were discarded and the micelle precipitate homogenized into a volume of 0.01 *M* $CaCl_2$ equivalent to the original skim milk volume using a Waring Blendor, modified to minimize foaming, and a 2 min. blending time. The pH was adjusted to 7.5 at 37° and micelles were centrifuged as before except that the centrifugation time was 60 minutes.

The micelle precipitate from each liter of skim milk was brought into solution by adding 600 ml. of 0.07 *M* potassium citrate. The temperature was maintained at about 8° and the pH at 6.5 by occasional addition of 2 *M* sodium acetate buffer at pH 5.5. After a few hours an additional 35 ml. of 1.0 *M* potassium citrate was added and the preparation was dialyzed against 0.08 *M* sodium acetate for 72 hours at 5° using 3 change of 8 liters each of dialysate. Preparations of FCC so obtained were stored at −20°. They apparently keep well for weeks. One liter of skim milk will yield 19–21,000 O.D. units of FCC, depending on the source of milk.

Preparation of α_s-Caseins

Crude α_s-Casein.—The FCC equivalent to 1 liter of skim milk was cooled to 0 to 1° and sufficient 5 *M* $CaCl_2$ added dropwise to give a final concentration of 0.17 *M*. The pH was maintained at 7.0 with 2 *M* tris buffer. The mixture then was placed in the cold room at 4 to 5° and allowed to stand overnight. A somewhat gummy cake precipitated. The supernatant was decanted and the precipitate brought into solution in 500 ml. of 0.05 *M* potassium citrate, the pH being maintained at approximately 6.5 and the temperature at approximately 2°. The solution was diluted to 4 liters with distilled water and the temperature brought to 5 to 7°, when sufficient 5 *M* calcium chloride was added to give a final concentration of 0.07 *M*. The precipitate which formed immediately and settled rapidly on standing was redissolved in 500 ml. of 0.05 *M* potassium citrate. It was either shell frozen and stored as such or first dialyzed at 5° against distilled water at pH 7.4. Each liter of skim milk yields about 5,000 O.D. units of crude α_s-casein. This procedure was adopted since it was known that the solubility of Ca β-caseinate increases[4] whereas the interactions between α_s- and β-caseins decrease[22] as the temperature is lowered. According to starch gel analyses crude α_s-casein was free of κ-casein; κ-casein was discarded in the final supernatant, probably in some combination with α_s-caseins.

$\alpha_{s1,2}$-Caseins and α_s-Casein Fractions.—$\alpha_{s1,2}$-caseins were separated from α_s-casein fractions and other impurities on DEAE-cellulose in a solvent containing 4.5 *M* urea. Urea dissociates not only α_s-casein polymers but the interaction products of α_s-caseins with other caseins.[24] With the caseins in the monomeric state, column fractionation was carried out DEAE-cellulose columns 8.4 cm. in diameter and 5 cm. long at 13°. Prior to the application of protein solution the column was washed with 4.5 *M* urea containing 0.01 *M* imidazole at pH 7.0 (B—I) and then with B—I containing 0.14*M* sodium chloride (B—I 0.14).

The crude α_s-casein solution contained 1,000 O.D. units in about 125 ml. A volume containing 1,000 O.D. units was mixed with an equal volume of 9 *M* urea and an appropriate concentration of sodium chloride in B—I so that the final volume was 2 liters, the pH was 7.0 and the added ionic strength was 0.12 to 0.125.

The final solution was delivered to the column at a rate of 25 ml. per minute. The casein solution was followed by about 1 liter of B-I 0.14. During application and washing with B—I 0.14 about 100 O.D. of unadsorbed protein impurity were removed from this column (column a) without removing significant amounts of $\alpha_{s1,2}$-caseins or α_s-casein fractions. At the same time B-I 0.14 did not remove other impurities, which remained adsorbed. These impurities, along with small amounts of $\alpha_{s1,2}$-caseins, were desorbed by B-I 0.155. Approximately 3 liters were required, the effluent being discarded. Optical density thus removed was approximately 100 O.D. A more precise specification is difficult to make since the protein-DEAE cellulose interactions at the loading used are quite sensitive to ionic strengths near 0.15. The proteins which remained adsorbed to the column were $\alpha_{s1,2}$-caseins and those constituting the α_s-casein fractions.

The reasons for adopting the procedure which follows will be made clear later in this article when the properties of the α_s-casein fractions will be given more detailed consideration.

After treatment with B-I 0.155 the output of the original column (column a) was made the input to a second column (column b) having the same dimensions as the first. Column b was previously equilibrated with B-I 0.16. The optical density of the output of column b was monitored. When these adjustments had been made B-I 0.18 was introduced into column a. Approximately 600 ml. of B-I 0.18 entered column a before optical density was recorded emerging from column b, at which point the output of column b was collected, and collection was continued until the output optical density had decreased to 0.03, which required about 6 to 7 liters.

Recovery of $\alpha_{s1,2}$-caseins from this volume was achieved by diluting each liter of output with 500 ml. of distilled water and passing the resulting solution through a small DEAE column 4 cm. in diameter and 8 cm. long. Flow rates of 30 ml. per minute were used without loss of protein. $\alpha_{s1,2}$-Caseins were removed from this small column with about 300 ml. of B-I 0.5.

Six liters of B-I 0.18 does not remove all $\alpha_{s1,2}$-caseins from preparatory columns a and b. Most of the remaining $\alpha_{s1,2}$-caseins were removed by exhaustive washing with B-I 0.18. This treatment, which required about 12 liters of B-I 0.18, was maintained until the output optical density of column b had not changed during the passage of 1.5 liters of B-I 0.18($\pm$ 0.003 O.D.). The proteins remaining on the preparatory columns were α_s-casein fractions. These were removed from the columns with B-I 0.5. The protein from column a will be referred to as α_s(a)-casein fraction, and that from column b as α_s(b)-casein fraction.

Customarily preparations of α_s-caseins have been dialyzed to remove salt, dried from the frozen state and stored at −15°.

After removal of α_s-caseins the columns were washed with 1 liter quantities of B-I 0.5 and then with B-I 0.15. After every four runs a wash of B-I 0.5 containing 0.1 *M* sodium hydroxide was used, then B-I containing 1.0 *M* sodium chloride and finally by B-I 0.15 until the pH had returned to pH 7.

Each 1000 O.D. units of crude α_s-casein yields approximately 600 O.D. units of $\alpha_{s1,2}$-caseins, 130 O.D. units of α_s(a)- and 50 O.D. units of α_s(b)-casein fractions; thus each liter of skim milk produces about 3,000 O.D. units of $\alpha_{s1,2}$-caseins and 900 O.D. units of α_s-casein fractions. That considerable amounts of the α_s-caseins are lost during fractionation will be seen from the abundances of these caseins, discussed below.

The $\alpha_{s1,2}$-caseins resulting from the two column procedure, just described, have occasionally been subjected to an identical refractionation process, again using two columns. This second fractionation removes certain minor contaminants observed by starch gel analysis in some $\alpha_{s1,2}$-caseins prepared as described. The presence of these bands will become explicable when the column behavior of the α_s-casein fractions is considered.

$\alpha_{s1,2}$-Caseins

Starch Gel Analysis.—In standard acetic acid gels at loadings of 0.75 to 0.1 mg. per slot and a current density of 7

(23) P. H. von Hippel and D. F. Waugh, *J. Am. Chem. Soc.*, **77**, 4311 (1955).

(24) N. Burk and D. Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

milliamperes per sq. cm., $\alpha_{s1,2}$-caseins characteristically exhibited two sharp bands which accounted for the majority of the stain uptake. At an average distance of 60 mm. from the origin the two band centers were separated by a distance of 4 to 5 mm., each band being 2 to 3 mm. wide. The band widths and separations were roughly proportional to the distance moved; thus, under optimal conditions doublets were observed with as little as 20 mm. of band movement. A third faint band of higher mobility, sometimes present, decreased markedly if the $\alpha_{s1,2}$-caseins were recycled through the column preparative procedure.

In tris citrate gels $\alpha_{s1,2}$-caseins gave a single broad trailing band at higher loadings but at about 0.1 to 0.01 mg. per slot two sharp characteristic bands were observed. Under the conditions used by Wake and Baldwin[1] *i.e.*, when the front had moved 120 mm. beyond the slots, the two characteristic bands were at a distance of 103 mm. from the slots, separated by a distance of 2 to 4 mm. and were each about 2 mm. wide. A third faint trailing band decreased in intensity with recycling.

That the bands had different mobilities, thus eliminating the possibility that the doublet was the result of inhomogeneous delivery of a single component from the slot, was shown by two dimensional starch gel electrophoresis. In one experiment the starch gel contained 7% acetic acid (pH 3.4). The first dimension employed 8 milliamperes per sq. cm. for 24 hours at 4° and the second dimension, 7 milliamperes per sq. cm. for 24 hours. After development two single bands whose mobilities were different in both dimensions were observed.

In addition to the standard acetic acid gel doublets also have been observed in gels containing 6.5 *M* urea, 0.05 *M* HCl (pH 2.8); in gels containing 6.5 *M* urea with acetic acid concentrations varying from 1 to 8% (pH 3.3 to 4.3); and in gels containing 4.5 *M* urea, 2% acetic acid (pH 3.6). pH values in some cases were adjusted by adding ammonia. In the absence of acetic acid, *i.e.*, at pH 8.4, 0.05 *M* imidazole, 0.08 *M* NaCl, in 4.5 *M* urea, doublets are still clearly discernible.

Diffuse single bands were often observed, particularly under conditions where the pH of the gel might not have been adequately stabilized. It is significant, however, that a single sharp band has not so far been observed in any experiment.

Two obvious explanations for the occurrence of doublets were considered: dissociation of a single component[25] or the presence of two components. The molecular weight determined by light scattering was 27,300 ± 1,500 at pH 12 and ionic strengths between 0.4 and 1.2.[26] From analysis of C-terminal groups the molecular weight was determined to be 27,000 to 27,500 (see below). Attempts were therefore made to ascertain whether the particle weight in urea, under conditions analogous to those used in starch gel analysis, was appreciably less than 27,000.

Membrane Dialysis.—Certain sizes of dialysis tubing manufactured by the Visking Corporation are known to pass small protein molecules.[27] If the doublets observed in starch gels were due to dissociation of a single component of mol. wt. = 27,000, one of the sub-units would have had to have a molecular weight equal to or less than 13,600. This is close to the molecular weight of ribonuclease which, in the following experiments, was used for comparison. Dialyses were carried out in a cell with a Lucite core; the protein solution layer was approximately 0.2 cm. in thickness and 5 cm. long. These cells conveniently held 2.5 ml. The dialysate volume was 25 to 35 ml. A particular roll of 20/32 Visking tubing, now several years old, was found satisfactory. A new roll did not pass ribonuclease.

$\alpha_{s1,2}$-Caseins, at a level of 20 to 25 optical density units per cell, were dialyzed against 4.5, 6.5 and 8.5 *M* urea with or without the addition of 0.05 *M* sodium chloride at pH 8.4, at 4° or room temperature. Under these conditions clear doublets had been seen in starch gel electrophoresis. Appropriate blanks were required since dialysis tubing in the presence of urea releases significant optical density. In all experiments 2% of the protein O.D. dialyzed out during the first 16 to 24 hours after which no further optical density appeared during a period of 6 days. The expected amount of protein optical density was recovered from the dialysand on terminating each experiment.

The same cells were then washed and used with ribonuclease (Sigma 5 × crystallized)[28] at a level of 10 optical density units per cell. In 6.5 *M* urea, 0.05 *M* NaCl at pH 8.5, about 25% of the total optical density of the ribonuclease appeared in the dialysate during the first 24 hours. After 3 days 65% of the original material had passed through the membrane; much of the remaining material appeared to be non-dialyzable since the amount of protein in the dialysate had increased by only a few per cent. at 7 days.

Osmotic Pressure.—The $\alpha_{s1,2}$-casein doublet was resolved in starch gels in the presence of 6.5 *M* urea at pH 3.8 to 4.3. These conditions were suitable for osmotic pressure measurements, particularly since these pHs were close to the isoelectric point of the protein. The osmotic pressures of $\alpha_{s1,2}$-casein solutions were determined in 6.5 *M* urea, 0.1 *M* KCl, at pH 4.3–4.6. Concentrations varied from 0.33 to 1.03%, but no significant dependence of π/c values on concentration was observed over this range, *i.e.*, neither Donnan effects nor changes in association were detectable. The molecular weight, calculated from the average of twelve measurements, was 26,900 with an estimated accuracy of ± 2,000. When the five measurements above 0.6% protein were used the value was 27,500 ± 900.

Attempts at Sub-fractionation.—Membrane dialysis and osmotic pressure experiments suggested that the doublet observed in starch gels was due to two components. In the standard column preparative procedure $\alpha_{s1,2}$-caseins are obtained by passing 6 to 7 liters of B-I 0.18 through columns a and b when they are arranged in series. In one experiment this total effluent was collected in four sub-volumes such that each contained approximately the same amount of protein. The protein from each sub-volume gave the same doublet pattern using standard acetic acid starch-gel procedures. There was no demonstrable shift in band pattern as would be expected if the bands represented independent components partially resolved on DEAE-cellulose.

$\alpha_{s1,2}$-Caseins are removed quantitatively by DEAE-cellulose from a buffer containing 8 *M* urea, tris at 0.02 *M* and pH 8.5. With this as the starting solvent for column fractionation, protein is not desorbed until the ionic strength of added sodium chloride is 0.125 ± 0.005. A large column was equilibrated with the starting solvent and 500 O.D. of $\alpha_{s1,2}$-caseins applied. Sodium chloride was added to the starting solvent until protein O.D. just appeared in the effluent (added ionic strength 0.130). Buffer of this composition was passed through the column until 220 O.D. units had been removed. The effluent optical density remained below 0.1. Protein was recovered from this buffer, whose volume was 4 liters, and the protein remaining adsorbed was removed from the column in the usual way. The two resulting preparations were examined by acetic acid starch gel electrophoresis. Both gave typical $\alpha_{s1,2}$-casein doublets which could not be distinguished from each other or from the $\alpha_{s1,2}$-casein control.

Chemical Analyses.—The extinction coefficient (E 1%) of $\alpha_{s1,2}$-caseins was determined to be 10.1. Optical densities were measured on a Beckman DU Spectrophotometer. Since the protein is insoluble at its isoelectric point, dry weights cannot be determined by weighing aliquots of a solution which has been exhaustively dialyzed *vs.* water. Instead, $\alpha_{s1,2}$-caseins were dialyzed *vs.* 0.1 *M* or 0.15 *M* KCl, adjusted to pH 6 to 7 with NaOH, and aliquots of both protein and dialysate were dried to constant weight in a vacuum oven at 105°.

For nitrogen analyses the percentage protein in the samples was determined from optical densities. Dr. Nagy's N value was 14.8%, based on two analyses, and ours was 14.5%, based on six determinations.

The phosphorus content of $\alpha_{s1,2}$-casein was found to be 1.03% ± 0.07 based on nine determinations. No cysteic acid was detectable by amino acid analysis of the oxidized protein. The methionine and total sulfur were therefore expected to be equivalent. Attempts were made to determine total sulfur by the Carius method but the resulting values were variable in spite of prolonged dialysis of the protein. A sample of $\alpha_{s1,2}$-caseins was oxidized with performic

(25) D. A. Yphantis and D. F. Waugh, *Biochem. Biophys. Acta*, **26**, 218 (1957). The dissociation of insulin into monomer in pyridine-water and acetic acid-water solutions was demonstrated.

(26) P. Dreizen, R. Noble and D. F. Waugh, *J. Am. Chem. Soc.*, **84**, 4938 (1962).

(27) L. C. Craig, W. Konigsberg, A. Stracher and T. P. King, "Symposium on Protein Structure," A. Neuberger, editor, John Wiley and Sons, Inc., New York, N. Y., 1958, p. 104.

(28) Sigma Chemical Co., St. Louis, Missouri.

acid according to Hirs and hydrolyzed.[29] The neutral amino acids were determined using a Spinco Amino Acid analyzer and the total N in the hydrolysate was determined by Kjeldahl method. If the N content was taken as 14.7%, there was one mole of methionine sulfone per 10,000 g. of protein.

$\alpha_{s1,2}$-Caseins are free of sialic acid by the resorcinol test[16] and free of hexose by the orcinol test.[17]

The contents of tryptophan and tyrosine were calculated from the absorption spectrum in 0.1 *N* NaOH according to the method described by Beaven and Holiday.[30] Using a molecular weight of 27,300, 2.3 tryptophan and 11 tyrosine residues per mole were found.

Crude α_s-casein was found to be free of sialic acid but to contain cysteic acid, after performic acid oxidation, at a level corresponding to one mole of disulfide per 200,000 g. The presence of a cystine-containing contaminant and some of its characteristics were established as follows. Crude α_s-casein was treated at pH 6.8–7.0 with rennin, 30 units per ml., at room temperature. After several hours a small fraction of the material formed a precipitate which contained all of the disulfide. When a system of $\alpha_{s1,2}$-κ-casein complexes, was similarly treated with rennin in the absence of calcium, carbohydrate was split off and κ-casein was converted within a few seconds to para-κ-casein, which contains the disulfide of κ-casein. This para-κ-casein, insoluble by itself, was held in solution by $\alpha_{s1,2}$-caseins and finally precipitated only after several hours when these had been converted to para-$\alpha_{s1,2}$-caseins. The properties of the contaminant thus suggest that it is likewise a derivative of κ-casein.

C-Terminal Groups of $\alpha_{s1,2}$-Caseins

Digestion of $\alpha_{s1,2}$-caseins with carboxypeptidases was used in the investigation of terminal groups. When $\alpha_{s1,2}$-caseins were digested with carboxypeptidase A (CPA) at enzyme:substrate (E:S) ratios of 1–1000 and pH 8 at room temperature, tryptophan, tyrosine, and leucine were released within 5 minutes and no appreciable amounts of other amino acids were detected for several hours. After 20 hours some additional amino acids appeared. At smaller E:S ratios, tryptophan appeared first, whereas leucine and tyrosine were released later and at about the same time.

Quantitative determination of the freed tryptophan was used to establish a molecular weight for $\alpha_{s1,2}$-caseins, and quantitative studies of the release of tyrosine and leucine were undertaken in an effort to determine the number of polypeptide chains present. Carboxypeptidase B digestions were performed in order to test for the presence of basic C-terminal amino acids.

Tryptophan Release.—The tryptophan and tyrosine released by treatment of the whole protein with CPA were separated from each other and the other components of the digestion mixture by passage through a column containing G-25 Sephadex.[31]

The digests contained 60 to 100 mg. of protein in about 2 ml., and were applied directly to the top of the column, which was 2.3 cm. in diameter and 70 cm. long. The developing buffer was 0.01 *M* ammonium bicarbonate adjusted to pH 7.5 to 8.0. The purity of the separated tryptophan was established from its spectrum in 0.1 *N* NaOH, after suitable correction for column buffer blanks. Quantitative determination of tryptophan was made from the optical densities of solutions adjusted to 0.1 *N* in NaOH. The molar extinction coefficient at $\lambda = 280$ mμ in 0.1 *N* NaOH was taken to be 5400.[30] When appropriate amounts of tryptophan were added to $\alpha_{s1,2}$-caseins in the absence of CPA, or to the protein core remaining after previous Sephadex fractionation, recoveries of tryptophan were 96 and 99%, respectively.

The conditions of digestion and the amounts of tryptophan released as a function of E:S ratio are shown in Table I. Extrapolation of the data to zero substrate:enzyme ratio yields a value of 27,000 to 27,500 for the molecular weight.

A peptide with C-terminal tryptophan was isolated after cleavage of $\alpha_{s1,2}$-caseins with cyanogen bromide. This reagent splits the peptide chain at the carboxyl side of methionyl residues, producing new C-terminal homoserine

TABLE I

DIGESTION OF $\alpha_{s1,2}$-CASEINS WITH CARBOXYPEPTIDASE A AT *p*H 7.9, 25° AND 16 HR.

E:S	Tryptophan released
1:600	1 mole per 35,000 g.
1:60	1 mole per 31,500 g.
1:60	1 mole per 31,800 g.
1:43	1 mole per 30,000 g.
1:12	1 mole per 28,500 g.
1:12	1 mole per 27,600 g.

or homoserine lactone, and is reported to react only with methionine under acid conditions.[32] Treatment of a 5% solution of $\alpha_{s1,2}$-caseins overnight with a thirty-fold excess (based on 3 methionines per mole) of CNBr in 0.1 *N* HCl produced some dialyzable material, including a peptide containing tryptophan. This peptide was purified by paper electrophoresis at pHs 6.4 and 1.9; it was located by staining for tryptophan, as it gave either a pale yellow or no color with ninhydrin stain. After elution from the paper the peptide was subjected to CPA digestion and to acid hydrolysis. Only tryptophan was released by CPA. Leucine and proline were found after acid hydrolysis. These results allowed the assignment of the amino acids methionine, proline, leucine, and tryptophan to a sequence in which tryptophan was C-terminal. The specificity of CNBr cleavage implies that this tryptophan had been terminal in the whole protein. From the composition and behavior of the tryptophan-containing fragment it must be concluded that more than one C-terminal sequence occurs in the $\alpha_{s1,2}$-caseins. Digestion of the peptide chain would be expected to stop near proline,[33] and, indeed, no proline was found to be released from $\alpha_{s1,2}$-caseins in the present experiments. Only the amino acids tryptophan and leucine, therefore, could possibly be released from the proline-containing methionine. . . .tryptophan sequence. The failure of the isolated peptide to release any appreciably leucine during CPA digestion strongly suggests that this sequence is not actually the source of the leucine freed upon incubation of $\alpha_{s1,2}$-caseins with CPA. Clearly, since the peptide contains no tyrosine, tyrosine released from the whole protein must come from another site.

Tyrosine Release.—CPA splits tyrosine from the $\alpha_{s1,2}$-caseins more slowly than it releases tryptophan. After 24 hours about one-third as much tyrosine as tryptophan was found in the digest. Tyrosine was measured after Sephadex fractionation using a value of 1524[30] for its molar extinction coefficient in 0.1 *N* NaOH.

However, release of tyrosine in amounts equivalent to tryptophan was obtained by CPA digestion of appropriately modified $\alpha_{s1,2}$-caseins which were digested with trypsin. To simplify the separation of tryptophan and tyrosine from the digest, the average size of the tryptic peptides was increased by blocking the lysyl ϵ-amino groups.

A solution of 120 mg. of α_{s2}-casein in 5 ml. of 4.5 *M* urea was maintained at pH 9 in a pH stat. Two additions of 130 mg. each of acetimidoester-HCl (neutralized just before addition) were made at intervals of one hour. The modified protein was dialyzed and lyophilized. Acetimidoester reacts with the lysyl amino groups to convert them to amidino-groups.[34] Trypsin will not split at the carboxyl side of residues so modified; if the reaction is nearly complete, the resulting tryptic digest will contain principally peptides formed by cleavage at the carboxyl side of arginine. Amidinated $\alpha_{s1,2}$-caseins were digested with trypsin (1:200) at 37° for one hour at pH 9. CPA digestion was then carried out overnight at room temperature and pH 8 at 1:1000 E:S ratio. Tryptophan and tyrosine were separated from the peptides on Sephadex. The ratio of tryptophan to tyrosine was determined to be unity from optical densities in 0.1 *N* alkali.

Peptides from the tryptic digest of amidinated $\alpha_{s1,2}$-caseins have been partially resolved by paper electrophoresis at pH 9 in 6 *M* urea. After electrophoresis, urea was removed by washing in 95 to 98% acetone. A peptide con-

(29) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).

(30) G. H. Beavan and E. R. Holiday, "Advances in Protein Chemistry," M. L. Anson, K. Bailey and J. T. Edsall, Editors, Academic Press, Inc., New York, N. Y., 1952, p. 319.

(31) Obtained from Pharmacia Fine Chemicals, Inc., Rochester, Minnesota.

(32) E. Gross and B. Witkop, *J. Am. Chem. Soc.*, **83**, 1510 (1961).

(33) H. Neurath in "The Enzymes," P. Boyer, H. Lardy and K. Myrback, editors, Academic Press, Inc., New York, N. Y., 1960, p. 11.

(34) M. J. Hunter and M. L. Ludwig, *J. Am. Chem. Soc.*, **84**, in press (1962).

taining tyrosine but neither arginine nor tryptophan was located by appropriate staining of guide strips and was eluted. On treatment with CPA this peptide released only tyrosine.

Leucine Release.—Leucine, like tyrosine, was released more slowly than tryptophan from the whole protein. For example, after 2–4 hours, CPA digestion at E:S ratio 1:10,000 gave tryptophan:tyrosine:leucine ratios of 1.0:0.1:0.1. The amino acid values were determined by ninhydrin reaction on paper subsequent to one-dimensional separation. Leucine was released from the whole amidinated protein at a rate nearly equal to that of tryptophan but the release of tyrosine was incomplete. Thus during digestion with 1:1000 CPA the try:tyr:leu ratios after 3 hours were 1.0:0.15:0.9 and after 6 hours were 1.0:0.14:1.1.

Among the fragments formed in the course of CNBr treatment of $\alpha_{s1,2}$-caseins were some dialyzable peptides, one of which contained C-terminal tryptophan (see above). Treatment of the entire dialyzable fraction with CPA yielded only traces of leucine, whereas appreciable leucine was released by digestion of the non-dialyzable fraction with 1:5000 CPA for 4 hours. Tryptophan, tyrosine, and leucine were released from this fraction in molar ratios of 0.3:1.0:0.8 as determined by ninhydrin after two dimensional separation of amino acids.

Thus, at appropriate times leucine is released from the amidinated protein in greater amounts than tyrosine and from the non-dialyzable CNBr fraction in greater amounts than tryptophan. These results indicate that neither the chain terminating in tryptophan nor a chain terminating in tyrosine can be the sole source of the leucine released by the action of CPA. The evidence supports the presence of a C-terminal leucine.

Absence of Arginine and Lysine.—Digestion of $\alpha_{s1,2}$-caseins with CPB[35] which had not been treated with DFP released arginine and lysine within a few minutes at pH 8, room temperature and an E:S ratio of 1:1000. Several neutral amino acids also appeared.

Since arginine could be determined by the Sakaguchi reaction in the presence of other amino acids,[36] its release was followed quantitatively in dialysates of digestion mixtures (E:S ratio of 1:100). With the untreated enzyme arginine was continuously released and to the extent of several moles per mole protein in 66 hours. The splitting of so much arginine from a protein containing 6 to 8 arginines per mole[37] suggested that the arginine (and lysine) were released by CPB subsequent to endopeptidase action. This conclusion was supported by the findings that base was consumed at pH 8 during the course of the reaction, of the presence of 6 or 7 ninhydrin positive bands after electrophoresis at pH 9 in ammonium carbonate buffer and by a decrease by a factor of 10 in enzyme activity subsequent to DFP treatment.[38] Release curves extrapolated to the origin. For comparison, it may be noted that globin releases all of its C-terminal arginine in one to four hours on digestion with 1:100 CPB.[39] Finally it was found that lysine appeared after arginine. Diminution of arginine (or lysine) release to low levels after DFP treatment, accompanied by decreased endopeptidase activity, implies that if endopeptidase activity were eliminated altogether, no arginine or lysine would be found in the digest. However, it is not possible to rule out the presence of a C-terminal arginine or lysine which is released slowly compared to the rate at which new C-terminal groups appear.

Absence of Histidine.—According to Davie, Newman and Wilcox,[40] histidine is split from β-lactoglobulin by CPA at pH 9.2. At this pH, an enzyme:substrate ratio of 1:1000, and after 4 hours $\alpha_{s1,2}$-caseins yielded approximately the same distribution of amino acids observed during the usual digestion at pH 8. At the end of two days of digestion, histidine had not appeared, even in trace amounts.

Absence of Aspartic and Glutamic Acids.—According to Green and Stahmann[41] CPA at an enzyme to substrate ratio of 1:2, and pH 5.0 will split glutamic acid from polyglutamic acid. These conditions were difficult to meet with $\alpha_{s1,2}$-caseins since the protein was insoluble at pH 5. However, it could be kept in solution by adding 6.5 *M* urea to the solvent. In the presence of urea, at pH 5.0, and an E:S ratio of 1:100 small amounts of tryptophan and leucine appeared after 4 hours. These amounts were considerably increased after 2 days showing that the enzyme retained some activity during much of the 2-day period. Paper electrophoresis at 4 hours or 2 days failed to reveal even traces of glutamic or aspartic acids. CPA was shown to split polyaspartic acid slowly under the same conditions.

α_s-Casein Fractions

When the recovery of α_s-casein fractions was desired, columns a and b were exhaustively extracted either with B-I 0.18 or B-I 0.2 to remove $\alpha_{s1,2}$-caseins. Elution of the remaining protein produced four types of fractions, which for convenience are designated a (0.2), b (0.2), a (0.18) and b (0.18) in Fig. 1 and Table II. Whenever tested, these fractions were found to combine with κ-casein and to produce micelles in the presence of calcium or other divalent cations.[42] Over a period of time pooled batches of α_s-casein fractions have been collected and these have been examined, along with individual lots.

In standard acetic acid gels the four α_s-casein fractions gave mainly single diffuse bands, clearly of lower mobility than $\alpha_{s1,2}$-caseins, but extending into the $\alpha_{s1,2}$-casein region. The presence of a trailing material near the origin was indicative of κ-casein, and occasionally faint auxiliary bands were seen.

Tris-citrate gels revealed extensive heterogeneity. Figure 1 illustrates the results. All pooled samples contained a trailing material, nearest the origin, in the position of κ-casein,[5] and had obvious doublets in the region corresponding to α_{s1}- and α_{s2}-caseins. In addition to these there were present at least four band regions, numbered to the side in Fig. 1. Bands in regions 4 and 2 appear to be relatively more prominent in the α_s(b)-casein fraction and the band, or bands, in region 3 more prominent in the α_s(a)-casein fraction. Individual α_s(a)-casein fractions generally exhibit all bands observed in pooled fractions while band regions 1 and 3 are often unoccupied in α_s(b)-casein fractions.

α_s-Casein fractions have been treated with CPA 1:12 for 4 hours at room temperature and tyrosine and tryptophan separated by Sephadex fractionation. The results are summarized in Table II. Unlike the $\alpha_{s1,2}$-caseins, the $_s$-casein fractions characteristically yield more tyrosine than tryptophan. If the tryptophan is assumed to come entirely from $\alpha_{s1,2}$-caseins it can be calculated that these fractions contain 12 to 30% of $\alpha_{s1,2}$-caseins. Non-$\alpha_{s1,2}$-casein contents are given in the last line of Table II. These contents possibly include some κ-casein, as shown by Fig. 1, which might be as high as 10%.

TABLE II

TYROSINE AND TRYPTOPHAN PER 10^5 G. α_s-CASEIN FRACTIONS[a]

	a (0.2)	a (0.18)	b (0.2)	b (0.18)
Tyrosine, moles	4.5	4.2	3.6	3.6
Tryptophan	0.5	0.7	0.4	1.2
Grams non-$\alpha_{s1,2}$-caseins $\times 10^3$	87	82	88	70

[a] a (0.2), etc., refer to α_s-casein fractions obtained after column treatment with B—I(0.2), etc., to remove $\alpha_{s1,2}$-caseins.

Protein remaining in each fraction after correction for $\alpha_{s1,2}$- and κ-caseins may be divided by corresponding (moles tyrosine − moles tryptophan) values. Average molecular sizes of 20,000 to 24,000 result. These are well below the molecular weight of $\alpha_{s1,2}$-caseins and permit consideration of the possibility that components of α_s-casein fractions may be derivatives of $\alpha_{s1,2}$-caseins.

(35) Prepared according to Folk, *et al.*[11]

(36) H. Rosenberg, A. H. Ennor and J. F. Morrison, *Biochem. J.*, **63**, 153 (1956).

(37) Analysis of a single sample of hydrolyzed crude α_s-casein on the Spinco Amino Acid Analyzer gave a value of 6.4 arginines per 30,000 g. Earlier analysis of "α-casein" indicated the presence of about 8 arginine residues per 30,000 g.

(38) J. A. Gladner and J. E. Folk, *J. Biol. Chem.*, **231**, 393 (1958,.

(39) E. Antonine, J. Wyman, R. Zito, A. Rossi-Fanelli and A. Caputo, *ibid.*, **236**, PC60 (1961).

(40) E. W. Davie, C. R. Newman and P. E. Wilcox, *ibid.*, **234**, 2635 (1959).

(41) M. Green and M. A. Stahmann, *ibid.*, **197**, 771 (1952).

(42) R. W. Noble, Thesis Research, M.I.T.

α_s-Casein fractions differ in phosphorus content, characteristic values being 1.35% P for α_s(a)-fractions and 1.16 % P for α_s(b)-fractions. Correction for the presence of $\alpha_{s1,2}$- and κ-caseins obviously would make these values higher for the remaining proteins, possibly as high as 1.5 and 1.35% P respectively. Contamination with these fractions will increase phosphorus contents of $\alpha_{s1,2}$-, β- and κ-caseins.

Column Behavior of α_s-Caseins.—At low ionic strengths DEAE-cellulose columns adsorb most of the components present in crude α_s-casein and retain them despite extensive washing. The effluent optical density can be reduced to essentially zero and the protein then may be considered to be *permanently adsorbed. For each individual component the* amount so adsorbed decreases as the ionic strength increases until, at some critical ionic strength, the column capacity for such adsorption vanishes. Thereafter, capacity is a function of the equilibrium solution optical density.

A column fractionation procedure, of the type used here, operates ideally when an ionic strength can be chosen at which one component is eluted while other components remain adsorbed. In each case the maximum ionic strength consistent with this requirement would be chosen to minimize elution volume. The column characteristics of casein components do not permit ideal fractionation and have required the development of unusual procedures. In the following discussion of the behavior of the caseins the results of pertinent experiments have been calculated for a column one sq. cm. in area and five cm. long, although data were obtained with columns 3.8 to 55 sq. cm. in area.

The removal of impurities which adsorb less strongly than $\alpha_{s1,2}$-caseins, with a minimum loss of these caseins, presents a small problem which has been met by limiting the total load and by using B–I 0.155. At this ionic strength the column retains a large capacity for $\alpha_{s1,2}$-caseins and impurities may be completely removed provided that a sufficient volume of buffer be used. These impurities have a low effluent (equilibrium) optical density when appreciable amounts are adsorbed. By discarding impurities as they emerge from col. a, the two column procedure prevents their adsorption to the cellulose of the second column and thus avoids larger volumes of eluting buffer.

Two types of experiments show that equilibrium between $\alpha_{s1,2}$-caseins adsorbed and in solution is rapidly attained. Single columns were initially loaded with 12.7 O.D. units protein per sq. cm. at 13° and B–I 0.18 was used as the eluting buffer. Elution was carried out at a flow rate of 0.45 ml. per sq. cm. per min. to the point where the effluent O.D. was between 0.05 and 0.2. If flow then was stopped for periods of five minutes to 16 hours, no increase in effluent O.D. was observed when flow was re-established. Under the same conditions, if flow were increased to 1.0 ml. per sq. cm. per min. a slight comparative decrease in effluent O.D. was observed, suggesting that this flow rate was too rapid for column equilibration.

In the presence of B-I 0.14 the column retains permanently in excess of 15 O.D. units of $\alpha_{s1,2}$-caseins per sq. cm. This capacity decreases with increasing ionic strength and becomes nil at B-I 0.165 ± 0.005. However, at equilibrium solution optical densities near 0.01 and eluting ionic strengths as high as B-I 0.2, considerable quantities of $\alpha_{s1,2}$-caseins are adsorbed. For example, 1.06 O.D. units per sq. cm. remain adsorbed in the presence of B-I 0.18 and an equilibrium O.D. of 0.016. At added ionic strengths above 0.2 the equilibrium O.D. of $\alpha_{s1,2}$-caseins, relative to a particular amount adsorbed, increases rapidly. However, the elution characteristics of the components of the α_s-casein fractions effectively limit the ionic strength at which $\alpha_{s1,2}$-caseins may be harvested to values below 0.2, as will be seen, and for this reason large amounts of harvesting buffer are required: six liters for B-I 0.18.

The standard load was 18 O.D. units crude α_s-caseins per sq. cm. When $\alpha_{s1,2}$-caseins were to be removed prior to the recovery of α_s-casein fractions, for the series of washing buffers B-I 0.17, B-I 0.18, B-I 0.19 and B-I 0.2 we have used 25, 18, 13 and 10 liters, respectively. The gel patterns of Fig. 1 and the tryptophan values of Table II indicate that even these volumes, for B-I 0.18 and B-I 0.2, did not in fact remove $\alpha_{s1,2}$-caseins entirely. However, the amounts of α_s(a) and α_s(b)-casein fractions remained the same regardless of the choice of washing buffer, showing that the components of the α_s-casein fractions at standard load have significant equilibrium O.D. even in the presence of B-I 0.17. The transfer of about 0.9 O.D. unit per sq. cm. from column a to column b and the volume effecting this transfer gave average equilibrium optical densities for α_s-casein fractions of 0.002 for B-I 0.17 to 0.005 for B-I 0.2. This conclusion was *checked in another way. It was established that either* α_s-casein fraction is removed from B-I 0.2 by DEAE-cellulose and that washing of either fraction with B-I 0.2 to an effluent optical density just within 0.01 of zero leaves about 1.6 O.D. per sq. cm. adsorbed.

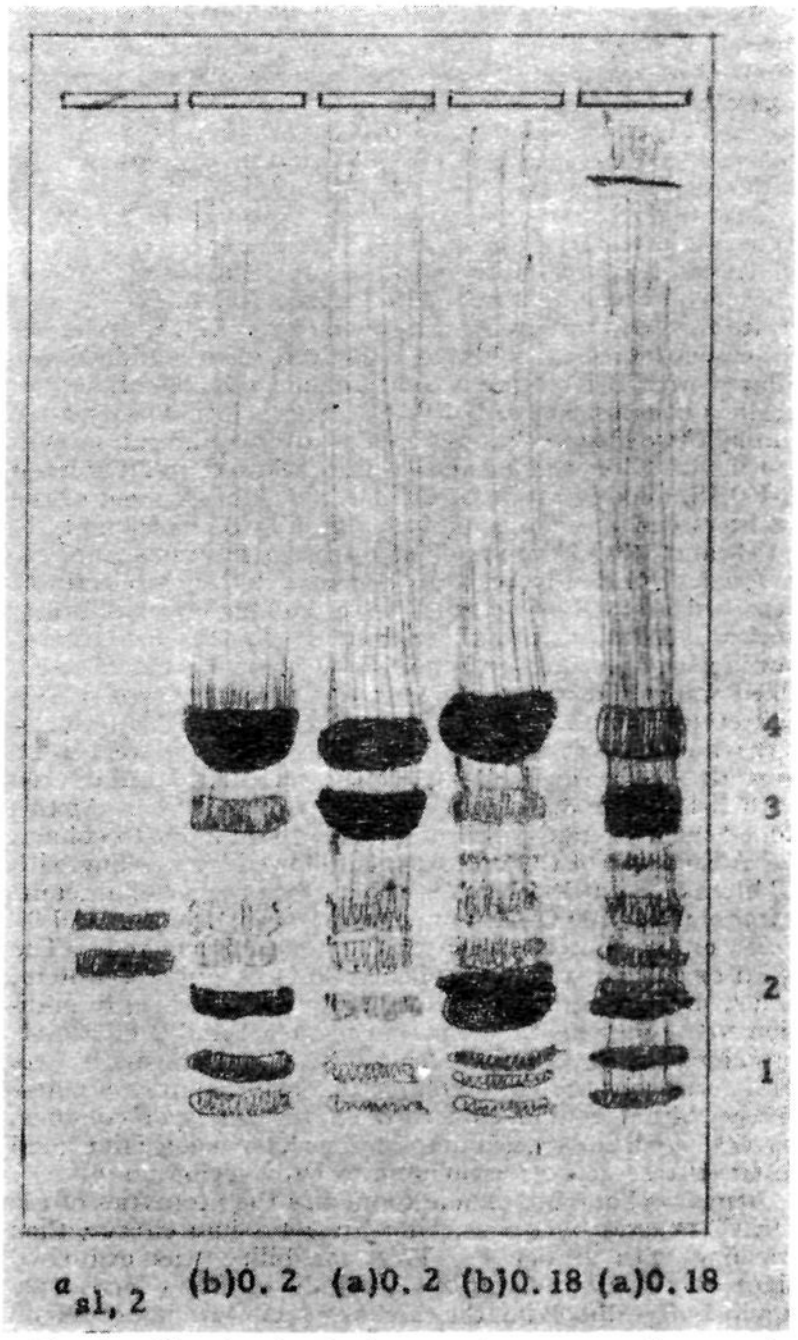

Fig. 1.—Sketch of tris-citrate gel patterns of α_s-casein fractions. The first column gives a comparison pattern of $\alpha_{s1,2}$-caseins. (b) 0.2, etc., specify the source of the fraction, column a or column b, and the added ionic strength of B-I used to remove $\alpha_{s1,2}$-caseins.

The possibility that competition for binding sites occurs among α_s-casein components, thus affecting the distribution of α_s-casein fractions, was examined. It was found, first, that both α_s-casein fractions are proportional to the load using the standard preparative procedure. In this procedure, of course, all of the protein to be fractionated is adsorbed to column a at the start. More definitive information was sought by applying the proteins in dilute solutions, at an ionic strength such that the major components, $\alpha_{s1,2}$-caseins, could cover only a small fraction of available sites. This was carried out as follows. Either 1000 or 500 O.D. units of crude α_s-caseins were applied to column a in the usual way, including washing with B-I 0.155. At this point all protein was removed from column a with 700 ml. of B-I 0.5 after which column a and column b were equilibrated with B-I 0.18. The proteins eluted ($\alpha_{s1,2}$-, α_s(a)- and α_s(b)-casein fractions) were diluted in such a way that they were in 10 liters of solvent equivalent to B-I 0.18. This solution was passed through the two columns in series and was followed by 8 liters of B-I 0.18. The 1000 O.D. units of crude α_s-casein yielded 124 O.D. units of α_s(a) and 47 O.D. units of

α_s(b)-casein fractions and the 500 O.D. units of crude α_s-caseins yielded 73 and 28 O.D. units, respectively. The levels of $\alpha_{s1,2}$-caseins or α_s-casein fractions are thus not important factors in the final distribution even when all protein is applied under conditions usually associated with the harvesting of $\alpha_{s1,2}$-caseins, *i.e.*, when the $\alpha_{s1,2}$-caseins are minimally adsorbed.

The several components present in α_s-casein fractions were found to have different equilibrium optical densities, which can account for the variation in composition of fractions as observed in starch gel patterns, Fig. 1. To demonstrate this, 64 O.D. units of α_s(a)- and 29 O.D. units of α_s-(b)-casein fractions, collected in B-I 0.5, were combined and diluted with B-I to give a total volume of 3,300 ml., equivalent in composition to B-I 0.18. This solution was passed through two clean columns in series after equilibration with B-I 0.18. The protein solution was followed by 20 liters of B-I 0.18, making the total volume 23.3 liters instead of the 18 liters customarily employed. Finally 61 O.D. units of α_s(a)- and 12 O.D. units of α_s(b)-casein fractions were recovered. Evidently, certain components were selectively desorbed and therefore must have had higher equilibrium optical densities. A final experiment was therefore carried out in which the effluent from column b was diluted with distilled water and any protein present was recovered with a collecting column. In this experiment 130 O.D. units of α_s(a)- and 52 O.D. units of α_s(b)-casein fractions were used. Just after collecting in B-I 0.5, they were mixed and diluted with B-I to give 3,300 ml., equivalent to B-I 0.15. At this added ionic strength all protein adsorbs initially to column a. Adsorption of the protein was followed by washing with 33 liters of B-I 0.18, which is about twice the usual amount. After eluting, 100 O.D. units of α_s(a)-casein fraction and 34 O.D. units of α_s(b)-casein fraction were recovered. The third or collecting column yielded 36 O.D. units of protein, which, by tris-citrate starch gel analysis contained in addition to $\alpha_{s1,2}$-caseins the bands, in regions 4 and 2 of Fig. 1, prominent in α_s(b)-casein fractions. Total recovery was 93%. Clearly the large volume of washing buffer progressively shifted components through the series of columns. Several of our $\alpha_{s1,2}$-casein preparations have had a faint band in tris-citrate gels corresponding to band region no. 4.

Attempts have been made to modify the properties of the DEAE column by treating it with potassium citrate, then washing with Buffer I. This possibility was examined since all recent preparations of crude α_s-casein have been applied after dissolving the calcium precipitate in potassium citrate, without removing the calcium and potassium citrates by dialysis. α_s(b)-Casein fraction was used as a test material. The same behavior pattern on treated or untreated columns was found at loadings of 1.5 O.D. units per sq. cm.

Abundances of the α_s-Caseins

The presence of a C-terminal tryptophan in $\alpha_{s1,2}$-caseins appears to be relatively unique, for this amino acid has been found not to be released from κ- or β-caseins, and thus may be used to estimate the abundance of $\alpha_{s1,2}$-caseins. The estimate may include small contributions from para-κ-casein, which will release tryptophan, and possibly from uncharacterized components, but the estimates are considered to be reliable to within 5% of the total FCC optical density.[43]

The tryptophan released from FCC by treatment with 1:100 CPA for twenty hours was measured after Sephadex resolution in each of two experiments. Assuming that $\alpha_{s1,2}$-caseins have a molecular weight of 27,000, an $E_{1\%}$ of 10.1, and are the sole source of tryptophan, it was calculated that $\alpha_{s1,2}$-caseins accounted for 41 and 46% of the total optical density.

The distribution of C-terminal tryptophan during fractionation and an estimate of the recovery of $\alpha_{s1,2}$-caseins in very crude α_s-casein fractions was obtained as follows. FCC was divided into three fractions: (1) precipitate which formed at 4° on standing overnight at 0.17 *M* Ca^{++}; (2) protein which subsequently was centrifuged down at 38° and (3) protein which remained in solution. Table III summarizes the average results from two experiments, in which each fraction was treated for 4 hours with 1:20 CPA at 25°. The short time reduced the probability that milk proteases might give rise to C-terminal tryptophan. Examination suggests that little of the $\alpha_{s1,2}$-caseins are carried into fraction 3 (Fraction-S, see 7) from which κ-casein has been obtained but that $\alpha_{s1,2}$-caseins are distributed almost equally between the first two fractions. Fraction 2, from which β-casein is normally prepared, contains less than half its optical density as β-casein.

The data of Table III and the results with FCC before fractionation suggest that 45% of the O.D. of FCC is $\alpha_{s1,2}$-caseins. The abundance by weight may be less, for β-casein has an extinction coefficient smaller than 10. Approximately 32% of the $\alpha_{s1,2}$-caseins in FCC were obtained in pure form.

TABLE III

TREATMENT BY CPA OF CRUDE FRACTIONS OBTAINED FROM FCC

Fraction	% total O.D. in fraction	% of $\alpha_{s1,2}$-caseins in fraction	$\alpha_{s1,2}$-caseins as % total O.D.
1	35	65	23
2	43	52	22
3	22	9	2
			47

The abundances of the α_s-casein fractions are more difficult to arrive at. The minimum abundances as derived from column fractionations are 500 O.D. units of α_s(a)-casein fraction and 240 O.D. units of α_s(b)-casein fraction per liter of skim milk. Combined, this is a minimum of 3.5% of the O.D. units present in FCC.

The average casein content of bovine milk, from proteins recovered as acid-precipitates, is given as 3% (see 43). The yield in optical density units reported here, 18 to 21,000 O.D. units per liter, is well below this average, even taking into account the low extinction coefficient of β-casein. This suggests that the abundances of the $\alpha_{s1,2}$-caseins be examined with respect to breed and individual animal. The milk used here came from individual Guernsey cows.

Discussion

The present work has resulted in the isolation of two caseins which together account for about 45% of the optical density of FCC. These have been designated $\alpha_{s1,2}$-caseins, the symbol α_{sn} being reserved for those caseins which interact with kappa casein at appropriate stoichiometric weight ratios, forming complexes in the absence of divalent cations and micelles clottable with rennin in the presence of divalent cations.[3,6] $\alpha_{s1,2}$-Caseins satisfy these criteria.

Starch gel analysis, the most discriminating tool now available for assaying the purity of the various caseins, indicates that the $\alpha_{s1,2}$-caseins are free of contaminants. Other preparations of "α-caseins" which have been subjected to analyses in tris-citrate starch gels (5) have proved to be multicomponent systems which presumably contain $\alpha_{s1,2}$-caseins as major moieties. Comparison with gels of Wake and Baldwin[5] suggests that $\alpha_{s1,2}$-caseins occur in the band region designated 1.07 to 1.14 by those authors.

The average molecular weight of $\alpha_{s1,2}$-caseins has been determined chemically by studies of terminal groups and physically by studies of osmotic pressure and light scattering. Measurement of the amount of C-terminal tryptophan released by carboxypeptidase gave a molecular weight of 27,000 to 27,500. The light scattering value is 27,300 ± 1500[26] and the osmotic pressure value (reported here) 26,820 ± 2,000. When osmotic pressures at protein concentrations above 6.0 mg. per ml. are used to calculate the molecular weight, the value is 27,500 ± 900. The good agreement among molecular weight determined in different solvents suggests that the

(43) T. L. McMeekin, "The Proteins," Vol. II, Part A, H. Neurath and K. Bailey, Editors, Academic Press, Inc., New York, N. Y., 1954, Ch. 16, p. 389.

value of 27,300 corresponds closely to the average monomer molecular weight of α_{s1}- and α_{s2}-caseins.

Some time ago Burk and Greenberg[24] obtained a value of 33,600 for whole casein from osmotic pressure measured in the presence of 6.5 *M* urea. Their casein was an acid precipitated material which had been washed with ether to remove lipids. Over 70% of the protein of whole casein can be accounted for by $\alpha_{s1,2}$-caseins and β-casein, which has a molecular weight near 24,000.[44] The molecular weight of Burk and Greenberg thus seems somewhat high and suggests the presence of higher molecular weight particles in whole casein. Direct comparisons are difficult to make, however, for whole caseins are known to be complex mixtures.

$\alpha_{s1,2}$-Caseins have been distinguished from one another by resolution on starch gels. At pH values of 2.8 to 4.3 and at pH 8.5 two bands of nearly identical mobility have been observed. The possibility that these bands represent, not two proteins, but subunits of a parent protein has been examined by studies of membrane dialysis and of osmotic pressure. Neither method gave evidence for dissociation of a 27,000 molecular weight monomer into sub-units. Moreover, the studies of casein phosphopeptides by Osterberg[45] suggest that subunits ought to be more readily differentiable than are the bands designated α_{s1} and α_{s2}. From phosphorus analyses it is calculated that there are nine phosphorus atoms per molecule in $\alpha_{s1,2}$-caseins. Seven of these phosphate groups have been located on a small stretch of polypeptide chain.[45] If dissociation of a single component were to occur, one of the sub-units would be expected to contain about 80% of the phosphorus. It would be surprising to find that the two materials had almost identical mobilities over a wide pH range and that they had the same adsorption–elution characteristics on DEAE-cellulose. The evidence, then, suggests that two components have been isolated together by the fractionation procedure.

The extent to which to α_{s1}- and α_{s2}-casein components are similar in physical and chemical structure can be determined finally only when the components have been fractionated and analyzed. At this time a number of observations support the conclusion that the α_{s1}- and α_{s2}-caseins are closely related. Molecular weights derived from two physical methods and one chemical method are the same; each of the four terminal amino acids which has been studied can be isolated in yields of one mole per 27,000–30,000 g. of protein; the components have not so far been fractionated on DEAE-cellulose; and only small differences in electrophoretic mobility are detectable at pH's 3 to 4 and 8.5. The mobility differences do imply some structural difference between α_{s1}- and α_{s2}-caseins, and the total tryptophan content, which is 2.3 moles per mole of protein as calculated from adsorption spectra, suggests differences in amino acid composition. Finally, the variation in phosphorus content between preparations is more than would be expected from the precision of the method used.

Nevertheless, the components seem sufficiently similar to warrant the suggestion that these components are genetically related, especially since genetic variations have been demonstrated for other milk proteins.[46]

The occurrence of a pair of proteins suggested that the component of greater mobility on the alkaline side of the isoelectric point be termed α_{s1}-casein, that of lesser mobility be termed α_{s2}-casein, and that the mixture studied here be given the designation $\alpha_{s1,2}$-caseins.[47]

$\alpha_{s1,2}$-Caseins appear to have three C-terminal amino acids: tryptophan, tyrosine, and leucine. Tryptophan has been assigned a C-terminal position because it is the amino acid released most rapidly from the whole protein by carboxypeptidase. Isolation of a fragment containing C-terminal tryptophan after cleavage by CNBr confirmed this conclusion. Leucine is considered to be C-terminal on the basis that, under appropriate conditions, it is released in greater amounts than either tryptophan or tyrosine. The evidence for a C-terminal tyrosine is not as definitive. The peptide sequence terminating in tryptophan cannot release tyrosine. Tyrosine was released from tryptic digests of amidinated protein in amounts equivalent to tryptophan and from CNBr treated protein in quantities essentially equivalent to leucine. The existence of a sequence terminating in tyrosine–leucine cannot be eliminated by these data. The only contrary evidence is the presence of a peptide in tryptic digests of amidinated protein which yields tyrosine but no leucine on CPA digestion. However, the yield of this peptide was unknown since such a peptide could have been produced in small amounts by a chymotryptic contaminant acting on a tyrosyl bond, its presence cannot be accepted as proving the presence of a C-terminal tyrosine in the whole protein.

There are, however, at least two C-terminal groups per mole. Manson[49] using a preparation corresponding to the crude α_s-casein of our fractionation procedure, reports that the only N-terminal group present was arginine and that one mole of arginine was derived from 30,000 g. of protein. It seems most likely that there is only one N-terminal arginine per 27,300 g. of $\alpha_{s1,2}$-caseins, the blocking of at least one N-terminal group is therefore suggested. Acetyl derivatives are reported to carry out this function in TMV protein.[50]

In combination with κ-casein and appropriate cations α_s-casein fractions will form micelles, which

(44) R. A. Sullivan, M. M. Fitzpatrick, E. K. Stanton, R. Annino, G. Kissel and F. Palermiti, *Arch. Biochem. Biophys.*, **55**, 455 (1955).

(45) R. Osterberg, *Biochem. et Biophys. Acta*, **42**, 312 (1960).

(46) R. Aschaffenburg and J. Drewry, *Nature*, **180**, 376 (1957).

(47) In a preliminary report[46] we referred to the α_s-casein fractions as α_{s1}-casein. At the time the abstract was written we were not fully aware of the heterogeneity of the α_s-casein fractions. The present nomenclature is preferable since it identifies the major α_s-casein components with subscripts 1 and 2 and specifies these according to electrophoretic mobility in starch gels. At the time naming was considered several alternatives were apparent. Unfortunately most of these had been made unacceptable by the fact that they had been used to name what we now know to be gross mixtures of components. Hopefully, all of the components present in casein have now been revealed in tris-citrate gels.

(48) D. F. Waugh, M. L. Ludwig, P. Dreizen and R. Noble, Abstract International Biophysical Congress, Stockholm, Sweden, 1961, p. 196.

(49) N. Manson, *Arch. Biochem. Biophys.*, **95**, 336 (1961).

(50) K. Narita, *Biochem. Biophys. Acta*, **30**, 352 (1958).

will clot on the addition of rennin. According to Fig. 1 and Table II, α_s-casein fractions contain, in addition to κ-casein and $\alpha_{s1,2}$-caseins, five components. Which, if any, of these components in the pure state can combine with κ-casein and form micelles is not known. If caseins of appropriate properties are isolated from α_s-casein fractions it is suggested that they be termed α_{s3}-casein, etc.

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Light Scattering Studies of $\alpha_{s1,2}$-Caseins[1]

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Light scattering studies have been carried out on $\alpha_{s1,2}$-caseins, a preparation which accounts for 45% of the optical density of whole casein and consists of two similar components.[13] The components exist in monomeric state at pH 12, ionic strength 0.3 to 1.2 and room temperature. Under these conditions the refractive index increment is 0.181 ml. per g. The molecular weight of protein dialyzed at pH 12 using protein concentrations above 4 mg. per ml. was found to be 27,300 ± 1500 (95% confidence limits). The second virial coefficient was approximated by the equation $B = [17 - 17\Gamma/2] \times 10^{-4}$ mole ml./g.^{2}. Protein dissolved directly at pH 12 gave a molecular weight of 27,000 ± 1000. Unpredictable, usually irreversible, aggregation occurs under these conditions: (a) at ionic strengths below 0.3 at pH 12; (b) on interaction with a factor released by dialysis membranes at high pH, acting at protein concentrations below 3 mg. per ml.; (c) when a rapid pH–ionic strength increase is imposed on protein in solution at pH 7; (d) on surface denaturation caused by bubbling or foaming.

Introduction

Whole casein has been treated by a variety of preparative techniques each of which produces a major fraction easily precipitable with calcium ion and having a greater electrophoretic mobility than the remaining proteins. These major fractions have been referred to as α-caseins.[4-8] Wake and Baldwin, using tris-citrate starch gels, have demonstrated that the α-casein preparations so far described are multicomponent.[9]

Physico-chemical studies of molecular weight have been carried out on such preparations. In the recent analysis of McKenzie and Wake[10] on a material prepared by calcium and alcohol precipitation[11] and known to contain several components,[9] molecular weights from ultracentrifugation studies were reported to be between 24,000 and 27,600. Earlier studies by Gillespie and Waugh, reported by Waugh,[12] on an α_s-casein prepared by calcium precipitation and also known to be impure gave a molecular weight of 23,300.

Recently, a casein preparation, termed $\alpha_{s1,2}$-caseins, has been obtained in this Laboratory.[13] On either acetic acid or tris-citrate starch gel electrophoresis, two sharply defined bands of nearly the same electrophoretic mobility are observed. These bands have been associated with two components, each having the same C-terminal groups (tryptophan, leucine, and probably tyrosine), the same N-terminal group (arginine[14]) and, so far, identical adsorption characteristics on DEAE-cellulose. That the two components, which are present in nearly equal amounts, are similar and are probably small variations of the same fundamental structure is clear. The $\alpha_{s1,2}$-caseins account for 45% of the optical density of the total casein. They exist as polymers at pH 7. The $\alpha_{s1,2}$-caseins interact with κ-casein and the resulting complexes in the presence of divalent cations form micelles which clot on the addition of rennin.

In this paper are described light scattering studies intended to determine the monomer molecular weight of $\alpha_{s1,2}$-caseins. Reduction to monomers of the polymers that occur at pH 7 requires either the introduction of charge as at high pH or a dissociating solvent system such as urea. Under these conditions ultracentrifugal methods necessitate the measurement of partial specific volume for the solvent environment,[15] and either correction for charge effects at high pH[16] or for possible preferential solvent interactions in urea solution.[17] These difficulties suggested the use of light scattering in solutions at high pH. By so doing preferential solvent interactions are avoided and the technique inherently yields a molecular weight and an interaction coefficient.[18]

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(4) K. Linderstrøm-Lang and S. Kodama, *Compt. rend. trav. lab. Carlsberg*, **16**, No. 1 (1925).

(5) R. Warner, *J. Am. Chem. Soc.*, **66**, 1725 (1944).

(6) N. J. Hipp, M. L. Groves, J. H. Custer and T. L. McMeekin, *ibid.*, **72**, 4928 (1950).

(7) T. L. McMeekin, "The Proteins," Vol. II, Part A, H. Neurath and K. Bailey, editors. Academic Press, Inc., New York, N. Y., 1954, Ch. 16, p. 389.

(8) D. F. Waugh, M. Ludwig, J. M. Gillespie, J. Garnier, E. S. Kleiner and R. W. Noble, Jr., *Federation Proc.*, **19**, 337 (1960).

(9) R. G. Wake and R. L. Baldwin, *Biochim. et Biophys. Acta*, **47**, 225 (1961).

(10) H. A. McKenzie and R. G. Wake, *Australian J. Chem.*, **12**, 734 (1959).

(11) H. A. McKenzie and R. G. Wake, *ibid.*, **12**, 712 (1959).

(12) D. F. Waugh, *Discussions Faraday Soc.*, **25**, 186 (1958).

(13) D. F. Waugh, M. Ludwig, J. M. Gillespie, B. Melton, M. Foley and E. S. Kleiner, *J. Am. Chem. Soc.*, **84**, 4929 (1962).

(14) N. Manson, *Arch. Biochem. Biophys.*, **95**, 336 (1961).

(15) P. A. Charlwood, *J. Am. Chem. Soc.*, **79**, 776 (1957).

(16) J. S. Johnson, K. A. Kraus and G. Scatchard, *J. Phys. Chem.*, **58**, 1034 (1954).

(17) J. W. Williams, K. E. VanHolde, R. L. Baldwin and H. Fujita, *Chem. Revs.*, **58**, 715 (1958).

(18) W. H. Stockmayer, *J. Chem. Phys.*, **18**, 58 (1950).