SEPARATION OF $\kappa$- AND $\alpha_{s1}$-CASEINS
BY GEL FILTRATION

by

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ABSTRACT

Studies were conducted to observe the changes in gel filtration elution profiles of casein fractions due to temperature, pH, sodium dodecyl sulfate (SDS) concentrations, and salts. From these results, the optimum conditions for separation were: 1) elution with $5 \times 10^{-3}$ M borate buffer at pH 10 and room temperature to separate $\kappa$-casein from acid casein; 2) elution with $1 \times 10^{-2}$ M phosphate buffer at pH II and 4°C to separate $\kappa$-casein from skimmilk; 3) elution with $1 \times 10^{-3}$ M SDS solution at room temperature to separate $\kappa$-casein from acid casein and skimmilk; 4) elution with $5 \times 10^{-4}$ M SDS solution at room temperature to separate $\alpha_{s1}$-casein from acid casein.

Casein fractions were characterized for purity by acrylamide gel electrophoresis, sedimentation velocity centrifugation, stability of casein fractions in the presence of calcium ions, and sialic acid content.

Compared to chemical preparations of casein fractions a reasonably pure $\kappa$-casein fraction was obtained by gel filtration when eluted with buffers at pH 10 and pH II. Casein fractions obtained by SDS elution yielded $\alpha_{s1}$- and $\kappa$-caseins almost as pure as chemically prepared and purified caseins. However, considerable decrease in stabilizing ability was observed in $\kappa$-casein eluted with SDS solution. It is possible that the decrease in stability is due to a binding of SDS with casein as the sulfur content of casein
fractions prepared by the SDS methods was higher than that of chemically prepared casein fractions. There was slight variation in the sialic acid content of κ-casein obtained by the different methods of preparations.
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INTRODUCTION

Recent advances in technique in protein chemistry have enabled a more detailed study of the physico-chemical and biological properties of milk proteins. The discovery of $\kappa$-casein in casein fractions brought about great progress in the understanding of the casein micelle structure and its stability. Consequently, it became of considerable importance to obtain casein fractions in a more natural state in order to study their properties and functions in the milk casein micelles.

A number of methods has been reported for fractionation of the casein complex. Although some progress in the procedures and in the purity of preparations has been achieved most methods suffer from one or more of the following defects: the low recovery of $\kappa$-casein fractions, high contamination, drastic conditions, lengthy and tedious procedures, and costly operations.

Recently Yaguchi et al. (67) introduced a gel filtration method for the fractionation of $\kappa$-casein from acid casein. They succeeded in isolating $\kappa$-casein fractions from crude $\kappa$-casein in the presence of urea. Cheeseman (II) in his study on the interaction of bovine milk caseins with SDS suggested a possibility of isolation of $\kappa$-casein by gel filtration with SDS as an eluent.

In the present study attempts have been made to separate $\kappa$- and $\alpha_{S1}$-caseins under mild conditions by gel filtration. A borate buffer, a phosphate buffer, and a diluted SDS
solution instead of high concentration of urea solution were used as eluents. The isolated casein fractions were examined for stability in the presence of calcium ions, purity by acrylamide gel electrophoresis, by sedimentation velocity centrifugation, and for K-casein, the sialic acid content.
A. Preparation of Casein and Identification of its Fractions

In 1838, for the first time, Mulder (39) reported that protein could be precipitated from bovine milk by adding acids, for which a widely accepted method was established by Hammarsten (18) in 1883. Although various methods for separation of casein have been developed, acid precipitation is usually employed as a standard procedure to define milk casein. According to a definition established by ADSA (56), casein is described as a heterogeneous group of phosphoproteins precipitated as pH 4.6 and 20°C. However, McKenzie (35) showed that the precipitation was far from complete at 2°C and even at 20°C it was still incomplete.

It was more than a hundred years after Mulder's work that von Hippel and Waugh (59) established a new method for the preparation of casein without adjusting the pH from neutrality: This procedure consists of two major steps: 1. shifting essentially all casein into the micellar form by adding calcium ion and centrifuging the solution to collect the micelles; 2. removal of calcium from micelles by adding potassium oxalate and oxalic acid, removal of calcium oxalate by centrifugation and removal of excess potassium oxalate by dialysis. McKenzie et al. (35) studied the isolation of whole casein from milk by ammonium sulfate fractionation, a technique which is commonly used as a gentle method of fractionating proteins. Ammonium sulfate (260 g/liter)
was added to the skimmilk with mechanical stirring, over a period of 40 minutes. The precipitate was collected by centrifugation, and dissolved in water. The procedure was repeated and the final solution was dialyzed exhaustively against glass-distilled water.

Since Mellander's first observation of α-, β- and γ-peaks in acid casein by moving boundary electrophoresis (36), attempts have been made to fractionate casein. Warner (62) developed a procedure for separating α- and β-caseins based on the higher solubility of β-casein at pH 4.4 and 2°C. Hipp et al. (24) developed two fractionation procedures which were based on: 1. difference in solubility of α-, β-, and γ-caseins in 50% alcohol in the presence of salt, as well as in water with changes in pH and temperature, 2. difference in the solubility of each casein fraction in aqueous urea solutions near the isoelectric point. In the first method α-casein was insoluble in 50% alcohol at pH 5.7 in the presence of salt while β- and γ-caseins were soluble. In the second method α-casein was insoluble in 4.6M urea solution at a pH near the isoelectric point while β- and γ-caseins were soluble under these conditions.

It has been thought that α-casein was a single protein which forms the protective colloid for stabilization of casein micelles, and upon which the enzyme rennin acted (I3) until Waugh and von Hippel (64) found evidence for the presence of a new component. They centrifuged (45,000xG) skim-
milk after addition of 0.06M calcium chloride and obtained calcium caseinate gel (first cycle casein fraction). After removal of calcium ions from first cycle casein fraction, 0.25M calcium chloride was added to the fraction and obtained precipitate (second cycle casein fraction P). It was found that the addition of calcium to the second cycle casein fraction P, at concentrations markedly lower than those found in skimmilk, led invariably to the formation of a coarse, heavy precipitate. At the same time it was known that the addition of calcium to the first cycle casein led to the formation of stable micelles of skimmilk. Therefore, it seemed possible that a stabilizing factor, was removed during the separation of the second cycle fraction P. To test this possibility, the supernatant obtained after calcium addition and centrifugation, at the beginning of the second cycle, was freed of calcium ions by oxalate titration and combined with second cycle casein fraction P in various proportions. An appropriate mixture of the supernatant fluid and the fraction P yielded stable micelles on the addition of calcium in amounts similar to those found in skimmilk. The major casein in the supernatant, the second cycle casein fraction S, was named as K-casein.

B. Preparation of K-Casein

Since this initial work by Waugh and von Hippel, there has been considerable interest in the properties of the K-casein fraction and a number of methods have been suggested
for its preparation. Wake (61) succeeded in the isolation of $\kappa$-casein containing only slight impurities from second cycle casein fraction S. Subsequently, McKenzie and Wake (34) improved this method which was a combination of the alcohol fractionation method of Hipp et al. and calcium precipitation method of Waugh and von Hippel. Meanwhile, for separation of purer $\kappa$-casein, Swaisgood and Brunner (54) attempted to utilize the finding of Hipp et al. that iso-electric casein is soluble in concentrated urea solution and the finding of Wake, (60) and Nitschmann and Beeby (44), that the primary scission product of the action of rennin on $\kappa$-casein, glycomacropeptide, is soluble in 12% trichloroacetic acid solution. They postulated $\kappa$-casein would remain soluble in concentrated urea solutions upon the addition of 12% trichloroacetic acid. The prepared $\kappa$-casein from whole casein at 4 C, contained 77% by weight of $\kappa$-casein. Cheese-man (10) prepared a mixture of $\kappa$- and $\alpha_{s1}$ -casein from acid casein by the urea fractionation and removed $\alpha_{s1}$-casein by addition of calcium. Hill (20) prepared crude $\kappa$-casein by modification of Waugh and von Hippel's method using a DEAE cellulose column for the purification of the crude $\kappa$-casein.

These and a number of other methods (27, 57, 58) for the preparation of $\kappa$-casein are in general tedious and time consuming. Zittle (71) and Zittle and Custer (73) proposed a method for preparation and purification of $\kappa$-casein, which has been most commonly used because of its relative
simplicity and high yield. Acid casein was dissolved in 6.6M urea solution and sulfuric acid was added to bring the pH to I.3. The precipitate which contains $\alpha_{s_1}$- and $\beta$-caseins was filtered and filtrate collected. The $\kappa$-casein in the filtrate was precipitated by the addition of ammonium sulfate. Subsequent alcohol precipitation produced an electrophoretically pure $\kappa$-casein retaining the stabilizing ability for $\alpha_{s_1}$-casein against precipitation with calcium chloride.

All procedures discussed so far are more or less a modification or a combination of one or more of the basic methods of Hipp et al. (alcohol or urea) (24), of Warner (solubility at pH 4.0 and 4 C.) (62) and of Waugh and von Hippel (calcium) (64).

C. Preparation of $\alpha_{s_1}$-Casein

Zittle (69) and Zittle and Custer (73) modified urea procedure of Hipp et al. in which extra NaCl was added to dissociate the $\alpha_{s_1}$-casein complex. Starch gel electrophoresis in urea showed that there was considerable contamination by $\beta$- and $\kappa$-casein. Therefore further alcohol fractionation was necessary for purification. Schmidt and Payens (52) separated crude $\alpha_{s_1}$-casein by means of a urea method subsequent to the calcium precipitation method. As a purification method column electrophoresis was used. Thompson and Kiddy (55) also used calcium precipitation and urea methods. Crude $\alpha_{s_1}$-casein was precipitated by diluting
a 5% casein solution in 6.6M urea to a final urea concentration of 3.3M at pH 4.6-4.8. This procedure was repeated twice and the precipitate dissolved in water by adjusting the pH to 7.2 and cooled to 2-4 °C. Four molar calcium chloride was added slowly to yield a final calcium concentration of casein solution of 0.4M. After repeating the calcium fractionation step followed by the removal of calcium, the crude $\alpha_{s_1}$-casein was purified by alcohol fractionation. In addition to this procedure, DEAE cellulose was used for complete purification of $\alpha_{s_1}$-casein. Thus, most of the standard procedure for the separation and purification of $\alpha_{s_1}$-casein were used in Thompson and Kiddy's method. No contaminants were observed on starch gel electrophoregrams.

D. Preparation of Casein and Casein Fractions by Gel Filtration

Since 1959 when Porath and Flodin (48) introduced gel filtration for the separation of molecules according to their size, this method has been used in various fields of study, particularly for biochemical and clinical research as an efficient tool for separation and purification of proteins. This method depending on the molecular sieve effect of a cross-linked dextran, was first applied to milk by de Koning (I5). He isolated milk protein from low molecular weight constituents of milk with Sephadex G-50 at neutral pH. Morr et al. (37) and Hill et al. (22) fractionated milk protein by gel filtration on Sephadex G-100 into four fractions which
were identified by electrophoresis to be casein micelle, α-lactoalbumin, β-lactoglobulin and non-protein nitrogen-containing substances in this order of elution. Since casein is in a micelle form at neutral pH no separation of casein fractions was observed.

Fractionation of casein by gel filtration after dissociation of casein micelle by changing pH and temperature, and by introducing dissociating agents was attempted by Yaguchi and Tarassuck (67) using Sephadex G-100. Κ-Casein, relatively pure electrophoretically, was separated at the void volume position by eluting with 6M urea at pH 8.6. Later, in 1968, Yaguchi et al. (68) were successful in preparing pure Κ-casein on Sephadex G-150 with a column equilibrated with 0.005M Tris-citrate buffer, pH 8.6, containing 6M urea. Cheeseman (II) in 1968 also reported the separation of Κ-casein from other casein on a Sephadex G-200 column equilibrated with 0.02M sodium dodecylsulfate. These works suggested the possibility for isolation of casein fractions by gel filtration under mild experimental conditions.
METHODS AND MATERIALS

Preparation of \( \alpha'_s \) - and \( \kappa \)-Casein as Standards

Zittle and Custer's method (73) was used to prepare standard \( \alpha'_s \) - and \( \kappa \)-casein. Crude \( \alpha'_s \) - and \( \kappa \)-casein were further purified by alcohol fractionation.

Nitrogen Determination

The casein content of freeze dried samples was determined by measuring the nitrogen content of samples by the Kjeldahl method (2). A conversion factor of 6.38 from nitrogen to casein was applied.

Gel Filtration

Sephadex G-100 and G-150 purchased from Pharmacia Fine Chemicals, Inc., were used for this study. The gel was soaked in distilled water and kept in boiling water for 5 hours. After swelling, the gel was equilibrated with the solvent. Procedures for assembling the upward flow system and for packing the column described by Pharmacia Fine Chemicals, Inc. (53) were followed.

After preliminary tests, three different solvents were chosen for the preparation of \( \kappa \)-casein: \( 5 \times 10^{-3} \)M borate buffer pH 10 for the separation of \( \kappa \)-casein from acid casein (pH 10 method), \( 1 \times 10^{-2} \)M phosphate buffer pH 11 for the separation of \( \kappa \)-casein from skimmilk (pH 11 method), and \( 1 \times 10^{-3} \)M SDS solution for the separation of \( \kappa \)-casein from acid casein or skimmilk (SDS method). For the separation of
$\alpha_{s_1}$-casein, a $5 \times 10^{-4}$ M SDS solution was used (SDS method).

Optical density of the effluents was monitored at 280 nm with ISCO column monitor or Beckman DB spectrophotometer. Fractions containing $\alpha_{s_1}$- or $\kappa$-casein were collected and dialyzed against tap water overnight and against deionized water for additional two days in a cold room. Samples were pervaporized and freeze-dried.

**Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis was performed by a modification of Aschaffenburg's method (3). The gels were prepared by the following procedure; 12 g. acrylamide, 0.6 g. N,N'-methylenebisacrylamide and 32 g. of urea were dissolved in 0.1 M Tris-glycine buffer (pH 9.1) and made up to a final volume of 120 ml. This solution was filtered through No. 1 filter paper into a vacuum flask. After the deaeration, 0.34 ml. of 2-mercapto-ethanol, 1.0 ml. TMED (30% N,N,N',N'-tetramethylethylene diamine in 95% ethanol) and 1.0 ml. 10% ammonium persulfate were added to the solution, in this order, mixed gently and poured immediately into a mold, covered with a Perspex plate and weighed down to exclude all the air. The gel was allowed to stand for 30-60 min. to permit completion of the polymerization.

A horizontal electrophoresis apparatus, with double containers at either end of the gel, was used in all the polyacrylamide gel electrophoresis. A sodium chloride solution, 0.1 M, and 0.1 M Tris-glycine were placed in the
outer and the inner containers, respectively. These solutions were renewed for every run. The outer and inner containers were connected with eight folds of cheesecloth.

The gel plate was placed on the electrophoresis apparatus and equilibrated by running the current through the gel for 24 hours at 4 C. The amperage was maintained 20 milliamperes on a power supply.

Samples were prepared as follows: To 0.2 ml of skim-milk or to 10mg of dried caseins, 2 drops of 0.35M Tris-glycine pH 9.1, 1 drop of 2-mercaptoethanol, and 15 mg of urea were added and the samples dissolved completely. Strips of Whatman 3MM filter paper 1.5x10mm, which were soaked in the samples, and the excess blotted, were inserted in the slot on the equilibrated gel plate. The gel plate was covered with Saran film to prevent drying. Electrophoresis was carried out, under the same conditions as the equilibration of the gel, for 24 hours in a cold room. The gel removed from the mold, was stained in 0.2% amide black 10 B solution containing 45% methanol and 9.8% acetic acid for 5 minutes and then destained by washing in 10% acetic acid until a clear gel background was obtained.

**Ultracentrifugation**

Schlieren patterns of casein fractions were obtained in a Beckman L2-65B preparative ultracentrifuge at 20 C. with schlieren optics. A filled-epon single sector cell having 2° sector angle and 12mm thick center piece was used.
I% α_{s1}-casein solution was made by dissolving α_{s1}-casein in pH II glycine buffer (33). The buffer was prepared by diluting the following to I liter with water: 10.2 ml N NaOH, 9.8 ml IM glycine-IM NaCl, and 36 ml 5M NaCl. 0.1M sodium phosphate buffer pH 7.6 was used to make I% κ-casein solution. All sedimentation coefficients (S-values) reported are observed values and are uncorrected.

**Sulfur Determination**

The determination of sulfur in casein prepared by several methods was done by the method described by Bardsley and Lancaster (4).

Reagents:

1. Hydrochloric acid, 6N.

2. Extracting solution: Dissolve 4.6g of NaH_{2}PO_{4}·H_{2}O in I liter of 2N acetic acid.

3. Sodium bicarbonate, finely divided with no lumps.

4. Barium chloride, 20 to 60 mesh crystals.

5. Gum acacia solution: Dissolve 0.5g of powdered gum acacia in a mixture of 50ml of glacial acetic acid and 50ml of distilled water. The solution permits growth of microorganisms but may be used for several days if refrigerated.

6. Potassium sulfate, reagent grade.

Procedure:

Approximately 50 ml of sample was weighed and placed in a high-form porcelain crucible. The casein sample was
mixed with 1 g of sodium bicarbonate and incinerated at 500 C in a electric muffle furnace for 3 hours. After cooling the contents of the crucible were transferred to an Erlenmeyer flask. Ten milliliters of the extracting solution was used to rinse the crucible and was poured into the flask. An additional 10 ml of the extracting solution was added. After the reaction subsided, the contents of the flask were shaken for 30 minutes. The suspension was filtered through Whatman No.1 paper. Ten milliliters of the filtrate was pipetted into a 100 ml Erlenmeyer flask. One milliliter of gum acacia solution and 1 ml of 6 N HCl were added. The contents of the flask were swirled and 0.5 g of BaCl₂·2H₂O crystals were added to the flask. After 1 min standing the flask was again swirled frequently until the crystals were dissolved. Within 2 to 8 min the absorbance at 420 nm was read on a Spectronic 20.

A standard curve was obtained in the following manner. Reagent-grade K₂SO₄, 0.5434 g, was dissolved in the extracting solution containing 100 ppm of S. This solution was pipetted into 100 ml Erlenmeyer flasks in increments of 1 ml up to 10 ml. Extracting solution was added to make the volume 20 ml resulting in the concentrations up to 50 ppm in increments of 5 ppm. Ten grams of sodium bicarbonate was added to each flask. After the reaction subsided, the solutions were filtered. Ten milliliters of the filtrates were pipetted into 100 ml Erlenmeyer flasks and analyzed as described for unknown samples.
Determination of Sialic Acid

The determination of sialic acid was carried out by the thiobarbituric acid method of Warren (63). In preparation for this analysis about 20 mg of the K-casein samples were hydrolyzed with 0.5 ml of 0.1N sulfuric acid for 60 min. at 80°C. The protein solution was centrifuged and 0.2 ml of the supernatant was used for the assay. The following reagents were used:

1. sodium periodate (meta) 0.2M, in 9M phosphoric acid;
2. sodium arsenite, 10%, in a solution of 0.5M sodium sulfate 0.1N sulfuric acid; and
3. thiobarbituric acid, 0.6%, in 0.5M sodium sulfate.

Procedure:

To 0.2 ml of sample containing 3 to 15 μg of sialic acid, in glass-stoppered tubes, 0.1 ml of reagent I was added. The tubes were shaken and allowed to stand at room temperature for 20 min. After addition of 1 ml of reagent 2, tubes were shaken until the yellow-brown colour disappeared. The tubes were shaken after addition of 3 ml of reagent 3, heated in boiling water for 15 min, and cooled in running water for 5 minutes. A equal volume of cyclohexanone, 4.3 ml, was added to the tubes and the contents were mixed. The mixture of cyclohexanone phase was transferred to a cell to measure absorbances at 549 nm in a Beckman model DB spectrophotometer. Readings were made against
the blank obtained by carrying 0.2 ml water instead of a protein solution. A molecular extinction coefficient of sialic acid of 57,000 was used for calculation.

Stabilization of $\alpha_{s1}$-Casein with $K$-Casein

Zittle's method (70) was used to estimate: I. the stabilization of $\alpha_{s1}$-casein by $K$-casein prepared by three different methods (SDS $K$-casein, pH 10 $K$-casein and Zittle's $K$-casein); 2. the comparison of stability of SDS $\alpha_{s1}$-casein and Zittle's $\alpha_{s1}$-casein with Zittle's $K$-casein against calcium ions.

1. I.5 ml of 0.5% $\alpha_{s1}$-casein and I ml of 0.02M imidazol buffer, pH 8, were mixed in I5-ml centrifuge tubes. Sufficient water was added to yield a final volume, after the subsequent additions, of 5.0 ml. The desired amount of 0.2% $K$-casein solution is added. To dissociate $K$-casein aggregates prior to the stability test, 0.02 ml of IN sodium hydroxide was added to the ice-cooled casein solution and immediately neutralized with 0.02 ml of IN hydrochloric acid. Finally I ml of 0.1M calcium chloride was added, to give a final pH of approximately 7.

2. I ml of 0.05% $K$-casein and I ml of 0.02M imidazol buffer at pH 8, were diluted with sufficient water to make a final volume, after subsequent additions, of 5 ml. The desired amount of 1% $\alpha_{s1}$-casein was added to make a $\alpha_{s1}/K$ ratio from 0 to 20, followed by the addition of I ml of
0.05M calcium chloride.

Both I and 2 preceded the following procedure. The mixture was stirred and kept in a 30 C water bath for 15 min. then centrifuged at about 3,000 g for 5 minutes. The supernatant fluid was clarified with one drop of IN sodium hydroxide and the casein in solution was determined from the absorbance at 280 nm. For experiment I, the amount of κ-casein used in the test was deducted from the estimated casein so as to measure the per cent of α_{s1}-casein contributing to the absorbance. For experiment 2, the per cent α_{s1}-casein in solution was calculated according to Beveridge (7).
RESULTS AND DISCUSSION

Gel Filtration

A. Effect of pH and Temperature on the Separation of \( \mathbf{K} \)-Casein

In preliminary tests, the effect of buffers of various pH, at various temperatures, on the separation of casein fractions was investigated. Although it is reported that Sephadex G-150 yielded the best resolution for whole casein (68), Sephadex G-100 was used for the preliminary experiment because of faster flow rates than Sephadex G-150 or G-200.

Some of the results are shown in Figure I. Elution profiles of acid casein from a column at alkaline pH revealed two peaks: the first peak eluted at the Vo position, containing mainly \( \mathbf{K} \)-casein, and the second peak was a mixture of \( \beta \)- and \( \alpha_s \)-casein. As pH was increased, the second peak shifted towards the low molecular weight fractions. Neither lower nor higher temperature during elution improved separation of peaks significantly. Reasonably pure \( \mathbf{K} \)-casein was eluted from Sephadex G-100 at the Vo position by elution with 0.005M borate at pH 10 and room temperature (Figure Iud). Therefore this condition was used for separation of \( \mathbf{K} \)-casein fraction from acid casein on Sephadex G-150 column of 2.5 by 85cm (Figure 2). The yield of \( \mathbf{K} \)-casein was about 10 mg from 0.6 g wet casein, which has approximately 75% moisture.
Fig. I. Effect of pH and temperature on elution patterns of acid casein obtained from Sephadex G-100 (2 by 40 cm). Condition:
(a) pH 9 and 45°C, (b) pH 9 and 4°C, (c) pH 10 and 5°C, (d) pH 10 and room temperature, (e) pH 10 and 45°C, (f) pH II and 4°C. Eluent: 0.005 M borate.
Fig. 2. Gel filtration of acid casein on Sephadex G-150 at room temperature. Eluent: 0.005 M borate, pH 10.
As an eluent one tenth normal sodium hydroxide and 2-4M acetic acid were not successful for this purpose.

The fraction range of Sephadex G-100 is for molecular weights of 4,000-150,000 for globular proteins, and 1,000-100,000 for polysaccharides. If caseins are in a micelle form, for which the molecular weight would be of the order of millions, all casein fractions should appear at the Vo position. However, if caseins are in a monomer form, it is difficult to separate each fraction by gel filtration since their molecular weight is similar. Increasing the pH to 10 or 11 for elution separated the casein fractions because of differences in the susceptibility of casein fractions to dissociation.

The caseins exist in a stable micelle structure in natural milk at a pH of approximately 6.5-6.9. When the pH is decreased to lower than 5.2, caseins start to precipitate, and at pH 4.6 all of the caseins are precipitated at room temperature, possibly due to an increase in the degree of hydrophobic interaction, in addition to the reduced repulsive forces.

When pH increases the net charges on the proteins increase resulting in an increase in electrostatic repulsion. In addition, the degree of hydration increases causing the decrease in hydrophobic interaction. It is reported that at pH 12 casein micelles are completely destroyed and casein assumes a monomeric form in solution (59).
It was found by Waugh (65) that cystine and cysteine in whole casein are concentrated in the \( K \)-casein fraction. Although Beeby (6) observed the presence of cysteine and not cystine in freshly prepared \( K \)-casein, the following reasons support the presence of cystine in \( K \)-casein. By gel electrophoresis, in the presence of 7M urea, \( K \)-casein appears as a smear band. Mackinlay and Wake (28) prepared a S-sulfo derivative of \( K \)-casein by a reaction of the protein with sodium sulfite in the presence of phenylmercuric hydroxide and obtained clear separation to two major bands and several minor bands. The sedimentation coefficient of \( K \)-casein treated with sodium sulfite was 9.1S while the corresponding value for \( K \)-casein was 13.2S. These results suggest that \( K \)-casein prepared by ordinary methods possesses -S-S-linkages and that either \( K \)-casein is cross-linked by oxidation of its -SH groups to create intermolecular -S-S- bonds during the preparation or, it possesses -S-S- linkages in native micelle structure.

The -S-S- bonds are unstable under the alkaline conditions, particularly at high temperatures. However, Mackinlay and Wake (28) observed that at pH 12, 94% of -S-S-bonds remained after 24 hours at 2 C, while at 20 C, 82% of -S-S- bonds were cleaved in 21 hours. Therefore it is possible that \( K \)-casein is still in a polymerized form through intermolecular -S-S- bonding at pH 10-11, while \( \beta \)- and \( \alpha_{SI} \)-caseins are more or less dissociated at this pH. This must
be the main reason for the separation of \( \kappa \)-casein from \( \alpha_{\text{s1}} \)- and \( \beta \)-caseins by using alkaline elution for gel filtration. Above pH 12, \(-S-S-\) bonding in \( \kappa \)-casein would be cleaved extensively (41) and no separation of casein fractions was observed. The results obtained from Sephadex columns by eluting with 0.1N NaOH revealed only one peak.

It is reported that caseins are highly hydrophobic compared to other proteins (8,23) and interact with each other partly through hydrophobic bonding. As Nemethy and Scheraga (43) stated the spontaneous formation of hydrophobic bonds in proteins is an endothermic process so that it is reasonable to expect a better separation at lower rather than at higher temperatures. The results indicate that hydrophobic interaction is not a main factor affecting the separation of the \( \kappa \)-casein fraction in this procedure.

B. Separation of \( \kappa \)-Casein from Skimmilk and the Effect of Calcium Ions.

Attempts have been made to separate \( \kappa \)-casein directly from skimmilk (Figure 3). No separation of \( \kappa \)-casein fraction was obtained by the condition used for acid casein. However, employing EDTA to clear skimmilk improved the separation of \( \kappa \)-casein considerably, especially at pH 10 as EDTA removes calcium ions from the casein complex system. At 4°C, and pH 11, a fair separation was obtained without using EDTA. The improvement in resolution by EDTA was not large, although there was a clear difference.
Fig. 3. Effect of calcium ions in skim milk on elution patterns obtained from Sephadex G-100 (2 by 40 cm). Conditions: a. pH 10 and room temperature without EDTA, b. pH 10 and 4°C with EDTA, c. pH II and 4°C without EDTA, d. pH II and 4°C with EDTA.
From the results, the column condition of pH II at 4°C with —_— EDTA was chosen for the separation of K-casein from skim milk. In order to minimize the denaturation of protein at alkaline pH by the prolonged elution time, the column was operated at low temperature. The elution profile from a Sephadex G-100 column, 4 by 142 cm, at pH II, is shown in Figure 4. About 50 mg of K-casein fraction was obtained from 30 ml of skim milk. The fractions A, B, C, D, and E were collected and retained for gel electrophoresis.

The considerable difference in the elution profiles of skim milk and acid casein, at pH IO and room temperature could be explained by the presence of calcium ions in skim milk. It is thought that calcium acts as a bridge between casein fractions in the native micellar structure which would bring the casein fractions close enough to have hydrophobic interaction. At room temperature hydrophobic interaction may occur between casein fractions, which resulted in one large peak at the Vo position at pH IO (Figure 3a). But at 4°C, less hydrophobic interactions are involved which may weaken the casein interaction. Thus, at low temperature, a fair separation could be obtained without using EDTA.

As soon as calcium ion are removed, the native micelle structure is destroyed, resulting in a decreased hydrophobic interaction since hydrophobic interaction is not sufficiently strong to hold casein molecules together in
Fig. 4. Gel filtration of skimmilk (30 ml) containing 600 mg of EDTA on Sephadex G-100 at 4°C. Eluent: $1 \times 10^{-2}$ M phosphate, pH II, with $5 \times 10^{-3}$ M EDTA.
the absence of a calcium bridge. This might produce the same condition as was observed for acid casein. As a result, we could obtain a similar elution pattern for skim-milk as for acid casein, from a Sephadex G-100 column.

It is not recommended to use high alkaline pH in order to avoid denaturation which, however, was not detectable during gel filtration by the pH IO and II methods. This method can provide edible \(K\)-casein for practical application.

C. Separation of \(K\)-Casein by Elution with SDS Solution and the Effect of SDS Concentration

Cheeseman (II) reported, in his study on the interaction of bovine milk caseins with detergents, that when the detergent was mixed with a buffer, relatively pure \(K\)-casein was obtained on Sephadex G-200. We intended to improve this method for practical applications of \(K\)-casein separated directly from skim-milk, or from acid casein, by gel filtration under mild conditions.

The effect of pH and concentration of detergent on casein micelles in the column was studied to find the best condition to fractionate \(K\)-casein. The effect of SDS concentration is shown in Figure 5. At a SDS concentration of \(2 \times 10^{-4} \text{M}\), two main peaks were obtained at the Vo and Vt positions from acid casein. No distinct peak was observed at a SDS concentration of \(1 \times 10^{-4} \text{M}\) and the absorbance at 280 nm was still high far beyond the calculated Vt position.
When SDS concentration increased to $1 \times 10^{-3}$ M the height of the second peak gradually decreased, becoming wider and approaching the Vo position. Finally at a SDS concentration of $1 \times 10^{-2}$ M, the two peaks came very close contaminating the first peak with $\alpha_{s1}$- and $\beta$- caseins to a greater extent than at lower SDS concentrations.

Acrylamide gel electrophoretograms indicated that $K$-casein was eluted at Vo position at all concentrations of SDS except lower than $2 \times 10^{-4}$ M. Reasonably pure $\alpha_{s1}$-casein was obtained in the later half of the second peak at $5 \times 10^{-4}$ M SDS. It was found that $1 \times 10^{-3}$ M of SDS concentration was most efficient for separation of $K$-casein. Approximately 60 mg of $K$-casein were obtained from 30 ml of skimmilk on a 4 by 142 cm column.

An interesting phenomenon observed in this experiment was a great increase in elution volume of casein fractions at very low SDS concentrations. When casein aggregates are partially dissociated, $K$-casein is eluted at the Vo position, if it was not reduced, and $\alpha_{s1}$- and $\beta$-caseins appear between the Vo and Vt position on a Sephadex G-100 in the presence of urea. But results showed that the casein fractions were retarded. To explain this difference in elution volume two possibilities must be taken into consideration. One is the effect of SDS on casein molecules, and the other is the effect of SDS on Sephadex gel matrix.

A unique character of the SDS as a denaturant is its
Fig. 5. Effect of SDS concentration in eluent on elution patterns of acid casein obtained from Sephadex G-100. Eluent: 
a. 2x10^{-4} M SDS, b. 5x10^{-4} M SDS, c. 1x10^{-3} M SDS, d. 5x10^{-3} M SDS, and e. 1x10^{-2} M SDS.
ability to produce drastic conformational changes at remarkably low concentrations while other denaturants such as guanidine hydrochloride, urea, inorganic salts and various simple organic substances induce conformational changes only at relatively high concentrations. Recently, Reynolds et al. (50) reported from a study on binding of SDS by bovine serum albumin, that the driving forces for unfolding brought about by SDS binding may be one or a combination of the following: (a) electrostatic repulsion between the charges of bound species, including the net charge of the protein; (b) penetration of the hydrocarbon tail into the apolar regions of the protein and the resultant replacement of conformation-stabilizing segment-segment interactions by ligand-segment interactions; (c) binding-induced changes in the protein hydrogen ion equilibrium resulting in an increase in electrostatic repulsion between charged species which are an intrinsic part of the primary protein structure; and that there is a favorable ratio of the number of binding sites and association constants in the native form to those in the unfolded form.

Payens and Markwijk (45) and Payens and Schmidt (46) suggested from the results of physico-chemical studies on the properties of the self-associating systems of the caseins, that hydrophobic binding is probably important in aggregation. Cheeseman (12) demonstrated the dissociation of casein
aggregates with SDS by means of sedimentation velocity. A fluorescence study of \( \kappa \)-casein interacted with SDS showed the decrease in hydrophobicity of \( \kappa \)-casein with increasing SDS concentration \((I4)\). This information suggests that SDS surrounds casein aggregates and is bound to hydrophobic sites between interacting casein molecules dissociating the casein aggregates. Due to the binding of SDS on casein molecules some configurational changes occur and the properties of casein differ from those of untreated casein.

Herries et al. \((I9)\) discussed the properties of Sephadex G-25 in the presence of SDS and stated that monomer units of SDS were adsorbed to the gel matrix considerably but SDS at concentrations above critical micelle concentration were excluded from the interior of the gel particles and behaved qualitatively as would be expected for a reversibly polymerizing system.

One possible reason for the delay in elution of casein fractions could be in an interaction of casein with the gel. Since SDS is absorbed on the gel as well as bound to casein molecules SDS on the gel and on hydrophobic sites of SDS-casein complex may attract each other resulting in a marked increase in elution volume. When certain amount of SDS are bound to protein through hydrophobic interactions the negative charge of the SDS-casein complex increases and counteracts the hydrophobic interaction which maintains casein aggregates. Thus at lower concentration of SDS hydrophobicity is strong enough to link proteins on gel, however, by in-
creasing SDS concentration electrostatic repulsive forces increase decreasing the hydrophobicity, and releasing the casein from the gel.

An increase of SDS concentration from 0.0001M to 0.0005M started separation of casein fractions on Sephadex gel columns. \( \kappa \)- and \( \alpha_{s1} \)-caseins were eluted at the Vo position and at an elution volume very close to \( V_t \), respectively. Separation of \( \kappa \)-casein from other fractions may be explained by intermolecular \(-S-S-\) bonds maintaining \( \kappa \)-casein molecule larger than others (II). Cheeseman reported that the maximum binding of SDS per mole of casein was largest in \( \beta \)-casein. However, no significant difference in sedimentation coefficient among the three major casein fractions was detected. In the presence of SDS, \( \alpha_{s1} \)-casein may have the strongest hydrophobic binding sites of the three different casein fractions since Cheeseman reported that the hydrophobic intermolecular bonding is the strongest in \( \alpha_{s1} \)-casein (I2). Thus, the complex, SDS-\( \alpha_{s1} \)-casein, probably interacts more strongly with the gel than other fractions enabling the separation of \( \alpha_{s1} \)-casein from others on the column.

D. Effect of Buffer Salts on SDS-gel Filtration

By adding buffer salts the pH of the SDS solution was changed and stabilized. The elution profiles with 0.001M SDS of which pH was stabilized with 0.005M phosphate, are indicated in Figure 6. The clearest separation of the \( \kappa \)-
Fig. 6. Effect of salts in SDS solution on elution patterns of skimmilk obtained from Sephadex G-100 (2 by 90 cm). Eluted with $5 \times 10^{-3}$ M phosphate buffer, pH 6 (a), pH 7 (b), and pH 8 (c), containing EDTA.
casein peak was obtained at pH 6. Addition of buffer salts did not improve the resolution and the SDS solution without buffer salts was used for elution thereafter. Unless the pH deviated from 6 considerably no adjustment was intended throughout this study. An increase in the ionic strength in SDS solution may antagonize dissociation of caseins with SDS. An increase of pH increased electrostatic repulsive force and decreased hydrophobicity. Therefore, casein-SDS complex can no longer react with gel, and there is only a sieving effect left in the gel.

Polyacrylamide Gel Electrophoresis

Since Raymond and Wang (49) found that genetic variants of milk protein can be identified conveniently by using polyacrylamide gels as the supporting medium, these gels have been used frequently instead of starch gels for the identification of genetic variants and subfractions. At present acrylamide gel electrophoresis is one of the best methods for determination of the purity of casein fractions. Consequently, in this study casein fractions from gel filtrations were examined by this method for their purity, and to establish the distribution of casein fractions on the elution patterns.

A separation of \( \kappa \)-casein by the pH 10 method is shown in Figure 7. Fraction A was a \( \kappa \)-casein with a slight contamination of \( \alpha_{s_1} \)-casein and fraction B was a mixture of \( \alpha_{s_1} \) and \( \beta \)-caseins with a slight \( \kappa \)-casein contamination.
Fig. 7. Acrylamide gel electrophoretogram of fractions of Fig. 2. W indicates whole casein.

Fig. 8. Acrylamide gel electrophoretogram of fractions of Fig. 4.
Figure 8 shows the increasing contamination in $\kappa$-casein fraction by the pH II method as the elution progressed. Fraction A was $\kappa$-casein of high purity while fraction E is the most heavily contaminated. Compared to the result of Yaguchi (67) obtained at pH 8 at 4℃ on Sephadex G-200, the purity of $\kappa$-casein on the gel electrophoretogram and the resolution of the $\kappa$-casein peak on Sephadex G-150 by the pH IO method were considerably improved. Yaguchi separated $\kappa$-casein directly from skim milk with a large amount of $\alpha_{s1}$- and $\beta$-caseins contamination. Our pH II method produced quite pure $\kappa$-casein.

Figure 9 shows electrophoretograms of fractions obtained by the $5 \times 10^{-4}$M (a), $1 \times 10^{-3}$M (b), $1 \times 10^{-2}$M (c) SDS methods. $\kappa$-Casein was in fraction A and fraction B in all three gels. Fraction A (c) was more highly contaminated with $\alpha_{s1}$- and $\beta$-caseins than fraction A (a). The lower the concentration of SDS used the purer the $\kappa$-casein obtained. Cheeseman (II) reported that fairly pure $\kappa$-casein was obtained by Sephadex G-200 gel filtration with an eluent of 0.05M Tris-HCl buffer of pH 7.6 containing 0.001M EDTA and 0.02M SDS. The same condition was repeated with Sephadex G-100 without successful separation of $\kappa$-casein. The condition we selected for the separation of $\alpha_{s1}$- and $\kappa$-caseins yielded a better resolution on Sephadex G-100 column and fairly pure $\alpha_{s1}$- and $\kappa$-casein bands on the gel electrophoretogram.
Fig. 9. Acrylamide gel electrophoretograms of fractions from Fig. 5b (a), 5c (b), and 5e (c).
Fig. 10. Acrylamide gel electrophoretogram of K-casein prepared by Zittle's method (Z), pH IO method (A), pH II method (B), and SDS method (S). M indicates skimmilk, and W indicates whole casein.
Fig. II. Acrylamide gel electrophoretogram of $\alpha_{\text{s1}}$-casein prepared by Zittle's method (Z), and SDS method (S).
As is indicated in Figure 10 \( \kappa \)-casein by the pH IO and pH II methods contained a small amount of \( \alpha_{51} \)-casein. Almost no \( \alpha_{51} \)-casein contamination was observed in \( \kappa \)-casein by the SDS method. Compared to the \( \kappa \)-casein by Zittle's method, \( \kappa \)-casein by the pH IO and pH II methods was less pure but the purity of \( \kappa \)-casein by the SDS method was comparable with that by Zittle's method. The \( \beta \)-lactoglobulin in the \( \kappa \)-casein prepared by the pH II method was probably contamination from the previous run, due to premature application of the next sample of skim milk to the column.

A comparison of gel electrophoretogram of \( \alpha_{51} \)-casein obtained by Zittle's method and by the SDS method is indicated in Figure II. Less contamination of the \( \kappa \)-casein like fraction with some minor fast- and slow-moving bands was observed in \( \alpha_{51} \)-casein by the SDS method.

**Ultracentrifugation**

The ultracentrifuge patterns of \( \kappa \)-casein prepared by gel filtration are shown in Figure I2. A single peak was observed in Figure I2 a for \( \kappa \)-casein prepared by the pH IO method. But two very small peaks and one large peak were obtained with the SDS method in 0.1M phosphate buffer at pH 7. The respective sedimentation coefficients of the major peaks were 11.5 S and 9.5 S.
Fig. 12. Ultracentrifuge patterns of $\kappa$-casein prepared by pH 10 method (a) and SDS method (b).
The ultracentrifuge pattern of $\alpha_{\text{s1}}$-casein prepared by gel filtration is presented in Figure 13. A single peak was observed. The sedimentation coefficient in 0.1M glycine buffer at pH 11 was 1.31 S. The sedimentation coefficient of 1.23 S was obtained for $\alpha_{\text{s1}}$-casein by Zittle's method.

In sedimentation velocity experiments one can separate different types of molecules, depending on their physical chemical properties. As a result, in Figure 12 b, two very small peaks, a fast-sedimenting and a slow-sedimenting peak were observed indicating heterogeneity of the particles. While Figure 12 a and 13 showed that $\kappa$-casein by the pH 10 method and $\alpha_{\text{s1}}$-casein by the SDS method were ultracentrifugally pure.

The lower value of sedimentation coefficient of 9.5 S, obtained with the SDS eluted $\kappa$-casein, as compared to the 11.5 S by the pH 10 method, may be due to the presence of SDS in the casein. Cheeseman (11) reported the effect of SDS on the sedimentation rates, showing three different sedimenting peaks 3 S, 7 S, and 11 S at SDS concentrations greater than 40 moles to one mole of $\kappa$-casein. He suggested that $\kappa$-casein forms two sizes of aggregates and the one which has $S_{20}$ of 6 to 10 S was a complex formed by intermolecular disulphide bonding since reduced $\kappa$-casein showed only a 3 S component. Observation of three peaks including two minor peaks may be indicative of the same tendency reported by Cheeseman. The higher value of the sedimentation
Fig. I3. Ultracentrifuge pattern of $\alpha_s$-casein prepared by SDS method.
coefficient for $\alpha_{s1}$-casein by the SDS method, 1.31 S, as compared to 1.21 S for $\alpha_{s1}$-casein prepared by Zittle's method may be due to the binding of SDS to the $\alpha_{s1}$-casein.

**Sulfur Content in Casein**

In order to investigate whether SDS still remains on casein fractions after three days dialysis with tap water and deionized water, the sulfur content of casein fractions were determined (Table I). Sulfur content in casein fractions by SDS method were higher than for casein fractions by Zittle's method. SDS residues of 14 per monomer of $\kappa$-casein and 6.2 per monomer of $\alpha_{s1}$-casein were present after dialysis. The value of 2.9 residues of 1/2 cystine and methionine in $\kappa$-casein by Zittle's method and 4.7 residues of methionine in $\alpha_{s1}$-casein prepared by Zittle's method were very close to reported values.

The results conclusively demonstrate the presence of SDS residues in casein fractions. The binding forces between casein and SDS are generally assumed to be electrostatic forces or hydrophobic interactions. The effects of SDS on the properties of casein fractions were detected by gel filtration and ultracentrifugation.
Table I. Sulfur Content in Casein
(per monomer of casein fractions: M.W. 20,000 for \(\kappa\)-casein, 27,000 for \(\alpha_{s1}\)-casein)

<table>
<thead>
<tr>
<th>Casein fraction</th>
<th>Total sulfur (g)</th>
<th>SDS(residues)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\kappa)-casein</td>
<td>94</td>
<td>-</td>
</tr>
<tr>
<td>SDS (\kappa)-casein</td>
<td>527</td>
<td>14</td>
</tr>
<tr>
<td>(\alpha_{s1})-casein</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>SDS (\alpha_{s1})-casein</td>
<td>347</td>
<td>6.2</td>
</tr>
</tbody>
</table>

*Remaining SDS residues in casein fractions were calculated by subtracting S value derived from 1.2 and 1.8 residues of \(\frac{1}{2}\) cystine and methionine in \(\kappa\)-casein and 5 residues of methionine in \(\alpha_{s1}\)-casein.
Sialic Acid Content of $\kappa$-Casein

The sialic acid content of $\kappa$-casein prepared by gel filtration using three different eluents are shown in Table 2. The highest value was obtained for $\kappa$-casein obtained by the pH method and $\kappa$-casein by the pH 10 method revealed the lowest value. The $\kappa$-caseins obtained by Zittle's method and the SDS method did not differ markedly.

Almost all sialic acid in casein is contained in the $\kappa$-casein fraction with only slight amounts were found in other casein fractions (26,32). Therefore considering the contamination of other fractions in $\kappa$-casein preparation separated by the pH II method, pure $\kappa$-casein should give higher sialic acid content than 2.19% (Table 2). $\kappa$-casein by the pH 10 method considering their degree of contamination showed a close sialic acid content to $\kappa$-casein by the SDS and Zittle's method. About 15% loss of sialic acid was observed in $\kappa$-casein by pH 10, SDS and Zittle's methods. Sialic acid content of $\kappa$-casein reported in the literature varies considerably from 0.8 to 2.5 g of sialic acid in 100 g of $\kappa$-casein. The values obtained from our results are within this range.

Considerable numbers of papers have been published about sialic acid content in milk especially in $\kappa$-casein. The difference in sialic acid content between cows and human milk (I) and between cows and buffalo milks (17) has been reported. Mackinlay and Wake (29) found only slight variation in the $\kappa$-casein content of whole casein prepared...
Table II. Sialic Acid Content in $\kappa$-Casein

<table>
<thead>
<tr>
<th>Method</th>
<th>% sialic acid in $\kappa$-casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zittle</td>
<td>1.85</td>
</tr>
<tr>
<td>pH IO</td>
<td>1.65</td>
</tr>
<tr>
<td>pH II</td>
<td>2.19</td>
</tr>
<tr>
<td>SDS</td>
<td>1.89</td>
</tr>
<tr>
<td>Cheeseman (10)</td>
<td>0.79</td>
</tr>
<tr>
<td>Huang et al (25)</td>
<td>1.37</td>
</tr>
<tr>
<td>Cayen et al. (9)</td>
<td>2.20</td>
</tr>
<tr>
<td>Gupta (17)</td>
<td>2.50</td>
</tr>
</tbody>
</table>
from cow colostral milk on the first and fourth days, but noticeable variations in the proportion of \( K \)-casein subfractions containing different amount of sialic acid. Thus, there may be a quite variation of sialic acid content from milk to milk due to a variation of \( K \)-casein content in the milk or a variation of sialic acid content in \( K \)-casein.

It was also reported that there was a variation in the sialic acid content of \( K \)-casein prepared by different methods from the same milk (21, 32). According to Marier et al. (32), \( K \)-casein prepared by Swaisgood and Brunner's method contained 2.14% of sialic acid while \( K \)-casein prepared by McKenzie and Wake's method contained 1.25% of sialic acid.

Mackinlay and Wake (30), Mackinlay et al. (31), and Schmidt et al. (52) pointed out that the different mobilities for each \( K \)-casein subfractions by gel electrophoresis could be explained by the different contents of carbohydrates, particularly sialic acid in the subfractions. No sialic acid content in the slowest moving fraction and increase in sialic acid content with increase in the mobility on a gel were observed for the subfraction isolated by DEAE cellulose chromatography. Therefore it is possible that a certain preparation method has a preference for the fractions containing less sialic acid. Beeby (5) postulated that there are three different fractions; a cystine containing component, a sialic acid containing component and a
component containing both cystine and sialic acid, but unsufficient evidence has been presented to support this assumption. However, should this be the case, sialic acid contents may have been caused by loss of the sialic acid containing component during preparation.

It is possible that the comparatively low values of sialic acid content for $\kappa$-caseins prepared by the pH IO, SDS and Zittle's method is due to the loss of sialic acid during the preparation of acid casein; skimmilk was used directly for the pH II method. Hill (21) reported that sialic acid content was reduced about 20-30% during acid precipitation accounting for the difference in sialic acid in our preparations of $\kappa$-casein.

**Stabilization of $\alpha_{s_1}$-Casein by $\kappa$-Casein**

It is known that in natural cows milk, calcium sensitive $\alpha_{s_1}$-casein is stabilized by $\kappa$-casein with the $\alpha_{s_1}$-casein to $\kappa$-casein ratio of approximately 4:1. Since Zittle (70) proposed a method to measure stabilizing ability of $\kappa$-casein, this has been a standard method for evaluating $\kappa$-casein with regard to the ability to stabilize the calcium sensitive casein preventing precipitation in the presence of calcium chloride.

The $\kappa$-casein preparations obtained from a column with different eluents were tested for their stabilizing ability. Results are shown in Figure I4. The elution with SDS markedly reduced the ability of $\kappa$-casein to stabilize
Figure 1. The solubilization of $\alpha_{\text{s1}}$-casein using SDS and various pH methods. The samples were prepared by Zittle's method, pH 11 method, pH 10 method, pH II method, and SDS method, respectively. The graph shows the percentage of $\alpha_{\text{s1}}$-casein in solution as a function of pH and SDS concentration.
\( \alpha_{s_1} \)-casein. This may be due to the remaining SDS in \( \kappa \)-casein sample. A slight decrease in stabilizing ability of \( \kappa \)-casein by the pH 10 method could be attributed to the contamination with other fractions mainly \( \alpha_{s_1} \)-casein, which increases the amount of calcium sensitive fragments and decreases stabilizing fragments. Nakai (42) reported that \( \kappa \)-casein eluted with 0.2 N NaOH at 4°C from Sephadex G-200 column restored most of its ability to stabilize \( \alpha_{s_1} \)-casein while a 1% solution of \( \kappa \)-casein held at 25 and 37°C in 0.2 N NaOH lost its ability to stabilize \( \alpha_{s_1} \)-casein rapidly. Therefore the slightly lower stabilizing ability of \( \kappa \)-casein obtained by pH II method could partly be due to high pH. However this is probably not the case because no denatured bands were observed by gel electrophoresis. It is interesting to note that when \( \kappa \)-casein and \( \beta \)-lactoglobulin are heated they interact with each other and decreased the stabilization of \( \alpha_{s_1} \)-casein in the presence of 0.02 M CaCl\(_2\) (72). Since \( \kappa \)-casein prepared by pH II method contained a fair amount of \( \beta \)-lactoglobulin band as shown by electrophoresis, its presence may explains the slightly lower stabilizing ability of \( \kappa \)-casein obtained by the pH II method.

A slight increase in stability of casein, but no change in \( \kappa \)-casein, by 0.1 M SDS was reported by Nakai (40). However \( \kappa \)-casein prepared by the SDS method yielded considerably low stabilizing ability. Mosolov and Lushnikova (38) reported that the esterase activity of trypsin was
inhibited by dodecyl sulfate, but acylation of the ε-amino groups of the trypsin molecule protected the enzyme from inactivation by dodecyl sulfate. They suggested that ε-amino groups of proteins participate in the interaction between SDS and proteins by electrostatic forces. Woychik (66) found that blocking free amino groups of \( \kappa \)-casein resulted in the decrease of stabilizing ability of \( \kappa \)-casein. Pepper et al. (47) also reported that modification of amino groups in \( \kappa \)-casein which discharges their positive charges, decreases its stabilizing ability. Thus the evidences have been supplied for the importance of amino group for stabilizing ability of \( \kappa \)-casein and also for the SDS interaction with free amino groups in proteins by electrostatic forces. Therefore, the observed decrease in stability would appear to be due to the interaction of SDS and the free amino groups in the protein. The behavior of SDS in a protein solution may be different at concentration above the critical micelle concentration, which caused the regain of stabilizing ability of \( \kappa \)-casein, since Gibbs et al. (16) reported that above critical micelle concentration SDS sometimes has a stabilizing effect on protein.

\( \alpha_{s1} \)-Casein prepared by the SDS method was tested to evaluate its stability in the presence of calcium ions and \( \kappa \)-casein. Figure 15 shows that Zittle's \( \alpha_{s1} \)-casein was slightly more stable than \( \alpha_{s1} \)-casein by the SDS method. Compared with a significant decrease in the stabilizing ability of \( \kappa \)-casein by SDS, the \( \alpha_{s1} \)-casein was almost intact.
Fig. 15. Stabilization of $\alpha_{s_1}$-casein by $\kappa$-casein. $\alpha_{s_1}$-Caseins were prepared by Zittle's method (---) and SDS method (--.--).
SUMMARY

Several advantages of using Sephadex gel columns as a tool to prepare casein fractions have been discovered in this study. First, high purity was obtained for casein fractions as shown by electrophoresis and ultracentrifugation. Chemically prepared crude \( \kappa \)-casein usually contains more contamination with \( \alpha_s \)- and \( \beta \)-caseins. \( \alpha_s \)-and \( \kappa \)-caseins prepared by Zittle's method, which is most commonly used, sometimes contains sizable amounts of contamination. Consequently, fractionation procedures have to be repeated and further purification procedures are also necessary. Contamination of \( \beta \)-lactoglobulin by the pH II method may be eliminated by careful operation of the gel filtration. Stability test of \( \kappa \)-casein showed that \( \kappa \)-casein prepared by the SDS method possessed a decreased stabilizing ability possibly due to the bound SDS on \( \kappa \)-casein, as a high sulfur content in the \( \kappa \)-casein was observed. If SDS was bound to casein fractions, it would be necessary to remove SDS from them.

Second, the procedure is quite simple. The column could be handled semi-automatically and used repeatedly without losing resolving ability. It is easily recognized that simplicity and reproducibility of a procedure is very important because of the complexity in conventional chemical separations. The reusability of the same column and the reasonable reproducibility for the pH IO and II methods are
advantages to the separation. However, the fairly rapid decrease in elution rate for the SDS method is disadvantageous. Several peculiar properties of SDS on gel columns were unexplained in this study and remain for further investigation.

Third, separation of the K-casein fraction directly from skimmilk with a purity comparable to that obtained from acid casein is feasible. General methods for the separation of casein fractions are from acid casein.

Fourth, the separation can be done under relatively mild conditions. Compared to the urea-gel filtration method, the SDS method is mild and economical. The concentration of urea required for elution is very high, (6M), and urea is costly.

Limitations of this method are; I) requirement for large columns to collect the quantities of the casein fractions necessary for other studies, and 2) relative length of elution times.
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