ISOLATION AND PROPERTIES OF KAPPA-CASEIN, GLYCOMACROPEPTIDE AND PARA-KAPPA-CASEIN, INVOLVED IN REACTION WITH THE ENZYME RENNIN

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THESIS

This is to certify that the

thesis entitled

Isolation and Properties of Kappa-Casein, Glycomacropeptide and Para-Kappa-Casein, Involved in Reaction with the Enzyme Rennin

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Edward M. McCabe

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Hans A. Lillevik
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ABSTRACT

ISOLATION AND PROPERTIES OF KAPPA-CASEIN, GLYCOMACROPEPTIDE AND PARA-KAPPA-CASEIN INVOLVED IN REACTION WITH THE ENZYME RENNIN

by Edward Mathew McCabe

The casein micelle of cows' milk is composed of several protein components and these components may also be heterogeneous in their nature. Kappa-casein (κ-casein) is a protein which stabilizes one or more of the micellar components. The enzyme rennin has been shown to act only on κ-casein yielding at least two reaction products, para-kappa-casein (p-k-casein) and a glycomacropeptide (GMP). The purpose of this study was to elucidate some of the chemical and physical properties of these reaction products.

Purified k-casein extracted from acrylamide gels was found to have a sedimentation coefficient of 1.7 S and a molecular weight of 19,000 in 5 M guanidine 2-mercapto-ethanol (2-ME) solutions.

Polyacrylamide urea gel electrophoresis (PAGUE) + 2-ME patterns stained for carbohydrate contents showed that the entire kappa region contained glycoproteins rather than in selected areas.

The use of sodium chloride fractionation was found to produce para-kappa-like material without the action of the enzyme rennin. A 1% solution of this p-k-casein-like material had a sedimentation coefficient of 0.9 S.

The 12% (w/w) trichloroacetic acid (TCA) soluble reaction product was found to contain two non-carbohydrate protein polypeptides in addition to the GMP. The GMP and the non-carbohydrate polypeptide fractions each appeared as two bands which may represent the genetic polymorphism. Polyacrylamide gel electrophoresis (PAGE) in a pH 8.6 continuous buffer system showed that the polypeptide fraction migrated faster than the GMP. PAGE in pH 8.6 discontinuous buffer systems showed that both fractions migrated as a single band with the migrating front. Passage of the 12% TCA-soluble GMP preparation over a Bio-Gel P-2 column removed both of the non-carbohydrate polypeptide fractions. This gel filtration could easily be followed by monitoring the column eluate at 280 mμ. The purified GMP isolated in this study was found to have a molecular weight of 7,500 and contained per mole of GMP two moles each of phosphorous, hexose, hexosamine and sialic acid. One-half of the hexose was removed by alkaline hydrolysis through a beta-elimination type of reaction mechanism which cleaves O-glycosidic and certain phosphoester bonds. A 0.3% solution of GMP in 01. N NaCl was found to have an absorbance of 0.44 at 280 mμ and a strong absorbance at 260 mμ.

P-k-casein from rennin-treated kappa enriched preparations was purified by preparative polyacrylamide disc electrophoresis. The $S_{20,w}$ was 1.3 S and a molecular weight of 14,000 was obtained in 5 M guanidine + Cleland's reagent. Approach-to-equilibrium experiments indicated that the lightest component had a molecular weight of 13,600.

Amino acid analysis of p-k-casein was performed, employing an internal standard of norleucine. The molecular weight calculated from amino acid composition was 12,000. One mole of phosphorous and hexose each per mole of p-k-casein were found present.

Starch-urea gel electrophoresis (SUGE) of rennin-treated k-casein demonstrated that only one p-k-casein band was initially formed, however, after its purification two p-k-casein bands appeared.

The sum of the molecular weights obtained by sedimentation equilibrium analysis of the rennin reaction products were found to be 21,000-21,500 which agrees closely with the molecular weight similarly obtained for k-casein, i.e., 19,000.

The crystalline rennin enzyme employed in this study was found to contain a small amount of impurity as shown by SUGE. Rennin's sedimentation coefficient was found to be 3.2 S and the molecular weight as determined by equilibrium ultracentrifugations was 31,000.

SUGE indicated that α- and β-casein-enriched fractions were split into several components by the action of rennin. The rennin was considered to have removed any kappa which may be present in the α_s-κ and β-κ-casein complexes.

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By

Edward Mathew McCabe

A THESIS

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DEDICATION

This manuscript is dedicated to my wife, Misako Marjorie, and family, Harold Mathew, Kathryn Lynne, Diana Chiyo and Sandra Aiko.

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INTRODUCTION

Casein, as a recognizable protein, was first prepared by Mülder in 1838 and is classically defined as that protein which is precipitated from skim milk at a pH of 4.6. Casein has been studied quite extensively since Mülder's time, but its exact physical and chemical nature is still unknown.

Casein is known to exist as a complex system of colloidal micelles composed of numerous components, the exact number of which has not yet been determined. Two of these components, α- and β-casein represent a large fraction of the total casein.

Present in the α-casein portion is a calcium-insensitive protein called kappa-casein (k-casein) which has been shown to be the most specific substrate for the enzyme rennin.

The action of rennin on k-casein results in at least two products, a glycomacropeptide (GMP) and para-kappa-casein (p-k-casein). The GMP is soluble in 12% trichloroacetic acid (TCA) and has been extensively characterized. P-k-casein is a rather insoluble protein, a property which has contributed to the sparsity of information concerning the nature of this protein.

The original objective of this investigation was to study the mechanism of rennin action on kappa-casein. A secondary objective was the physical and chemical examination of the rennin reaction products.

The original objective required highly purified and chemically unaltered kappa-casein and this proved to be a monumental task requiring numerous preparations and a great deal of time. The investigation proceeded to the secondary objective and purified the reaction products obtained by the action of rennin on whole casein and enriched kappa-casein preparations.

While the glycomacropeptide had been studied rather extensively, it had not been studied with regard to purity or in any great depth. The study of para-kappa casein began by first obtaining the protein in a rather purified state and then proceeding to examine the physical and chemical characteristics.

The enzyme rennin was analyzed with regard to purity and ultracentrifuge characteristics. The effects of rennin on α- and β-enriched casein fractions were investigated by gel electrophoresis.

Techniques for obtaining purified products needed to be developed. Since p-k-casein is relatively insoluble in the usual solvent systems, the problem of selecting a solubilizing system was explored. The GMP in addition to being obtained in a purified state also needed some method for visually elucidating its purity.

HISTORICAL

Whole casein is the protein which is precipitated from skim milk at a pH of 4.6. It has been studied quite extensively for well over one hundred years and even to this date its exact physical and chemical nature remains a mystery.

Almost a century ago, Hammarsten (1877) posed the question; "Is there a substance in the milk and in the casein solutions able to dissolve casein calcium phosphate which will be destroyed by the rennet, and is cheese the only casein calcium phosphate made insoluble by destruction of this compound?" Hammarsten found that the process proceeded in at least two steps. The first of these was an enzymatic destruction of a hypothetical protective colloid or solubilizing agent leading to a slight change of the casein, followed by a nonenzymatic clot formation.

The solubility studies by Linderstrøm-Lang and Kodama (1925) clearly showed that casein was a mixture of proteins and suggested that the stability of the caseinate system was due to the protective colloid action of one of its components. The ultracentrifuge studies by Pedersen (1936) demonstrated that casein sedimented not as a single

component, but as a polydisperse mixture, and Mellander (1939) found by moving boundary electrophoresis three casein components (α-, β- and γ-). Thus the heterogeneity of casein was not only firmly established but also that it consisted of at least three major proteins termed α-, β- and γ-casein.

Nitschmann and Lehmann (1947) observed that when casein was treated with rennet it differed electrophoretically from untreated casein in that the α-casein peak split into two peaks.

Cherbuliez and Baudet (1950) were able to demonstrate that an α-casein solution coagulated when attacked by rennet.

Alais, Mocquot, Nitschmann and Zahler (1953) showed that the nitrogenous compounds of milk soluble in 12% TCA increased during rennet action and reached a maximum before coagulation occurred.

Thus a concept concerning the action of rennin on casein seemed to evolve and may be summarized in general as follows:

Primary phase--the destruction of the protective colloid,

Secondary phase--formation of a coagulum as a result of micellar association, and

Tertiary phase--general proteolytic action of rennin slowly hydrolyzing all casein components.

During the action of rennin on α-casein, Alais et al. (1953) observed that in addition to the non-protein-nitrogen soluble in 12% TCA, other fractions were released which were soluble at pH 4.7 and only partially soluble in TCA concentration below 12%. The release of non-protein-nitrogen was very rapid at first, but soon declined to a slow, constant rate. This latter rate was attributed to products released by general proteolysis during the tertiary phase. Alais (1956) believed that one component was present in the non-protein-nitrogen fraction to a greater amount than the others, and that it was a glycopeptide of relatively high molecular weight. Nitschmann and Henzi (1959) showed that a glycopeptide and four other components were present in the non-protein-nitrogen 12% TCA-soluble fraction following rennet treatment of α-casein.

One of the outstanding advances in casein chemistry came forth when Waugh and von Hipple (1956) dissociated α-casein into two components by the addition of calcium. A calcium-sensitive fraction which precipitated in 0.25 M Ca^{++} was termed α_s- and a calcium-insensitive fraction was called k-casein. They suggested that k-casein provided the primary stabilization action in the α_s-k-casein complex by diminishing the number of calcium-sensitive groups on the casein complex.

An improved method for the isolation of k-casein was first reported by Wake (1959a) and McKenzie and Wake

(1961) which enabled them to study the action of rennin on this and other casein fractions. Their work clearly demonstrated that the non-protein-nitrogen fraction, soluble in 12% TCA that was cleaved from whole casein during the initial action of rennin, came from the kappa fraction. When k-casein was treated with rennin it clotted either in the presence or absence of calcium ions. Their work along with similar results by Tsugo and Yamauchi (1959) supported the contention of Waugh and von Hipple (1956) that k-casein was the primary target for rennin action in the clotting phenomenon.

The specific action of rennin on k-casein appears to be the release of the GMP with the consequent formation of an insoluble material called p-k-casein.

The overall proteolysis reaction was formulated as follows:

$$\text{Kappa-casein} \xrightarrow[H_2O]{\text{rennin}} \text{para-kappa-casein} + \text{glycomacropeptide}$$

Additional and modified techniques for isolating k-casein were developed by numerous other investigators. The purity and heterogeneity of k-casein prepared in the different ways were examined by Wake and Baldwin (1961); Neelin, Rose and Tessier (1962); Neelin (1962) and Garnier, Ribadeau and Gautreau (1962) with the result that all preparations were found to be heterogeneous.

The nature of the bond between the GMP and p-k-casein, which is severed by rennin during the primary phase, has been subject to much speculation. Nitschmann and Varin (1951) discussed peptide bonds, acid amide bonds, ester bonds, phosphoric amide bonds and bonds to guanidine as all being possibilities.

Mattenheimer, Nitschmann and Zahler (1952) demonstrated that rennin did not possess phosphatase activity, thereby reducing the probability that the rennin susceptible bond could be a phosphoester.

Wake (1959b) determined the N-terminal amino acids of k-casein and p-k-casein and reported both proteins had the same N-terminal residues, namely that of aspartic and glutamic acids. This observation provided evidence that no peptide bond is broken by rennin during the primary phase.

Jollés, Alais and Jollés (1962) were not able to demonstrate any N-terminal amino acid for k-casein, p-k-casein or the GMP, but did report the presence of some C-terminal amino acids. They found that k-casein and the GMP yielded serine, valine, threonine and alanine when treated with carboxypeptidase, while p-k-casein gave leucine and phenylalanine. This tended to suggest that rennin may break an ester bond occurring between phenylalanine within the p-k-casein and a hydroxyl group in serine, threonine or the carbohydrate moiety of the GMP.

In following the release of hydrogen ions during proteolysis by titration to a constant pH, Garnier, Mocquot and Brignon (1962) reported that one carboxyl group per mole of substrate was set free. This observation was proved more directly when Jollés, Alais and Jollés (1963) treated k-casein with $LiBH_4$. An ester bond was reduced and cleaved to yield p-k-casein, with phenylalaninol as the C-terminal group plus free GMP.

The hypothesis that an ester bond links p-k-casein to the GMP is not unequivocally accepted. Cheeseman, Rawitscher and Sturtevant (1963) could not detect the release of a proton during the action of rennin. They measured the heat of reaction of rennin on k-casein in L, tris (hydroxymethyl) aminomethane (Tris) and phosphate buffers. If a proton was released by ionization of the newly formed carboxyl group, when an ester bond is hydrolyzed, there should have been a much greater difference between heats of reactions in the Tris and phosphate buffers due to the much larger heat of ionization of the Tris.

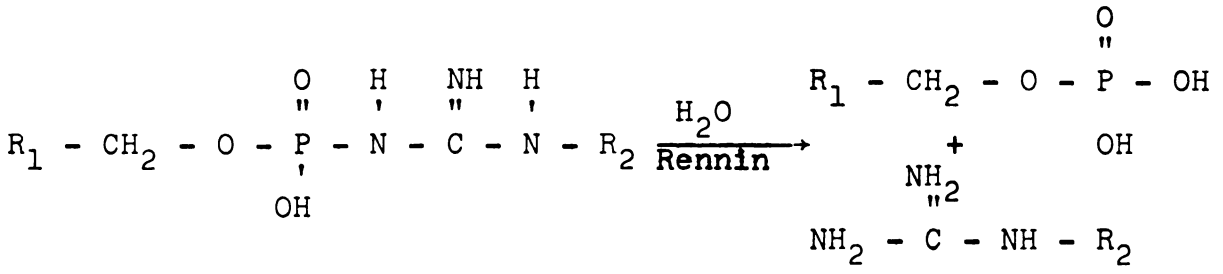
Neuraminidase can hydrolyze the sialic acid moiety from k-casein without much effect on its stabilizing power toward α_s-casein (Thompson and Pepper, 1962) or the action of rennin upon k-casein (Gibbons and Cheeseman, 1962). Thus it would seem unlikely that sialic acid could be involved in linking p-k-casein to GMP.

Beeby and Nitschmann (1963), while studying the effect of urea, pH and rennin on k-casein, suggested that

k-casein may not be a simple protein after all, but, instead, a complex which is stabilized by secondary bonds. The existence of a complex would then explain the electrophoretic heterogeneity observed in k-casein preparations under disaggregating conditions. They proposed that the first action of rennin on casein was the rapid disruption of the kappa complex by opening the secondary bonds responsible for its stability.

Lahav and Bahad (1964) studied the action of rennin on whole casein and on α-, β- and γ-casein and concluded that there may be several different calcium-insensitive fractions which are rennin sensitive, and each was principally associated with its own casein fraction α-, β- or γ-casein.

Dyachenko (1959) postulated that rennin cleaves the phosphoamide linkage and proposed the following reaction:



The phosphoamidase action of rennin on casein was investigated by Aiyar and Wallace (1964). There appears to be a direct correlation between the phosphorous released and the exposure of guanido groups of arginine as a result of rennin action on casein. They proposed that rennin

hydrolyzed a phosphodiamide bond and that the phosphorous was linked through guanido groups of arginine.

Hill (1964) using enzymatic digests found that casein micelles contained mainly cysteine and not cystine. Beeby (1964) was able to demonstrate that freshly prepared k-casein contained only cysteine. Delfour, Jolles, Alais and Jolles (1965) found that a very labile methionine residue appeared to be N-terminal on the GMP.

Several investigators (Neelin, 1964; Schmidt, 1964; and Woychik, 1964) demonstrated molecular polymorphism in reduced k-casein by means of gel electrophoresis. This polymorphism is based on the occurrence of one or two major gel bands in the reduced k-casein from the milk of individual cows. Additional heterogeneity was indicated by the presence of several electrophoretically discernible minor components in the reduced k-casein (MacKinlay and Wake, 1964; Schmidt, 1964; and Woychik, 1964). These investigators suggest that the major components represent genetic variants of the same protein and that the minor components arise by addition of various amounts of carbohydrate to the major components. The major components appear to lack carbohydrates, namely hexose and sialic acid, which are concentrated in the minor components causing them to migrate ahead of the major components (Purkayastha and Rose, 1965; and Woychik, Kalan and Noelken, 1966).

Woychik et al. (1966) designated the two types of k-casein as k-casein A and k-casein B; the major components of reduced k-casein A and reduced k-casein B as k-casein A-1 and k-casein B-1; and the minor components of reduced k-casein A and reduced k-casein B, consecutively in the order of increasing electrophoretic mobility as A-2, A-3, etc., and B-2, B-3 etc.

Utilizing S-carboxamidomethyl derivatives of k-casein A and k-casein B from the milk of individual cows to stabilize protein -SH groups, they were able to isolate chromatographically the major k-casein components A-1 and B-1. These major components were found to have a molecular weight of about 19,000 and appeared free of carbohydrates. The amino acid data revealed that component A-1 had one aspartic acid and one threonine residue more than B-1, while B-1 had one alanine and one isoleucine residue more than A-1. The amino acid composition of several of the minor components from the two types of k-casein was similar to, but not identical with, the major component with which the minor component was associated.

DeKoning, Van Rooijen and Kok (1966) were unable to demonstrate any differences in the amino acid composition of the GMP and the polypeptide from the k-casein genetic variant A. Likewise no amino acid variation was found in the same two moieties from the B variant. However, they were able to show that the GMP and polypeptide from k-casein A had one more aspartic acid and threonine residue than the

B variant, which in turn had one more alanine and isoleucine residue. These results confirm the work of Woychik et al. (1966) and demonstrate that the genetic polymorphism of k-casein is located in the peptide moiety cleaved off by rennin.

In starch-urea gel electrophoresis (SUGE), pH 8.6, p-k-casein migrates toward the cathode showing two major bands. Both MacKinlay, Hill and Wake (1966) and Woychik et al. (1966) demonstrated that each genetic variant of k-casein produces two p-k-casein bands, and propose that one p-k-casein band results from the minor components of k-casein associated with a given variant.

As noted earlier the GMP was discovered by Alais (1956) and Nitschmann, Wissmann and Henzi (1957) calculated its molecular weight to be about 8,000. Brunner and Thompson (1959) determined a number of its physical characteristics and calculated the molecular weight to be 15,000 from sedimentation and diffusion data. Both Wake (1959a) and Jolles, Alais and Jolles (1961) have obtained molecular weights of 8,000.

The amino acid composition of the GMP cleaved from k-casein by rennin was determined by Nitschmann and Beeby (1960) and found to be identical with that GMP cleaved from whole casein. Jollés and Alais (1960) and Jollés et al. (1961) also determined the amino acid composition of the GMP. It appears to them that the carbohydrate content of

the GMP may vary somewhat with the source of the peptide and method of preparation. The GMP is devoid of aromatic amino acids and arginine. It is relatively high in glutamic acid, threonine, proline and isoleucine and also contains phosphoserine.

P-k-casein has not been studied as closely as the GMP. Cerebulis, Custer and Zittle (1959) attempted to separate the insoluble material after the primary phase of rennin action, when whole casein and α-casein were used as a starting material. The colloidal chemical properties of p-k-casein were studied by Tsugo and Yamauchi (1959) and Yamauchi (1960). They found that neither the α_s-κ-casein complex or the β-κ-casein complex was split by rennet during the primary phase. This observation indicated that the casein micelles are intact after the primary phase and the only change is the loss of the protruding hydrophilic portion of the k-casein.

Amino acid analyses of p-k-casein have been reported by Jollés et al. (1963); Kalan and Woychik (1965); and DeKoning et al. (1966).

EXPERIMENTAL

Apparatus and Equipment

The raw pooled milk used in this study was obtained from the Michigan State University dairy barns and collected in a five- or ten-gallon stainless steel can. All procedures following the initial precipitation of whole casein were performed in plastic or pyrex containers. The milk was separated in a small (Model 9) DeLaval disc-type separator. The hydrogen ion concentration was measured with a Beckman Zeromatic pH meter equipped with a glass electrode. A Lightnin (Model L) mixer with a stainless steel shaft and blade was used to stir the casein solutions. Low-speed centrifugation was performed either in an International centrifuge with a capacity of 1500 ml or in a Servall type SS-1 centrifuge with a capacity of 400 ml. Free-boundary electrophoresis was performed in a Perkin-Elmer, Model 38-A Tiselius electrophoresis apparatus using circulating refrigerated water to maintain the bath temperature at 2° C. Buffer resistances were measured with an Industrial Instruments, Model RC, conductivity bridge.

A Plexiglas horizontal cell was used for starch-urea and polyacrylamide gel electrophoresis. A glass column

6 cm (I.D.) by 16 cm fitted with a course fritted glass filter at one end was used for preparative disc gel electrophoresis. A Savant Instrument Company power source (d.c.) was employed for all gel electrophoresis.

Sedimentation-velocity, sedimentation-equilibrium and diffusion studies were performed in a Spinco Model E Analytical Ultracentrifuge equipped with a RTIC temperature control unit and phase plate as a Schlieren diaphragm. A capillary-type synthetic boundary cell was used for sedimentation-velocity studies and initial concentration determinations. A capillary-type double-sector synthetic boundary cell was used for equilibrium experiments, initial concentrations and diffusion studies. A regular double-sector cell was used in approach-to-equilibrium experiments. Twelve millimeter cells, equipped with quartz windows, were used in all determinations. Centrifugations were performed in an An-D Duralumin rotor and a General Electric AH-6 Mercury Lamp served as the light source.

The Schlieren patterns were recorded on Kodak Metallographic glass plates and measured with a Nikon Model 6 Shadowgraph microcomparator. This microcomparator stage is able to traverse 25 mm on the Y-axis and 50 mm along the X-axis and can be read directly to 0.002 mm.

For precise weighings, a Cahn Gram Electrobalance was used. It has a total capacity of 1 g on the maximum range and 1 mg on the minimum range. The balance setting of the wheatstone bridge reads to four places on all ranges.

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

Prior to analysis protein samples were dried in a temperature controlled Cenco vacuum oven overnight at 25° C. and about one mm of mercury.

The ultra violet absorption of the eluates at 280 mu was measured by a monitor made by Gilson Medical Electronics and recorded on a recording milliammeter manufactured by Esterline Angus Instrument Company.

A Waring Blendor manufactured by the Waring Products Company was used to disintegrate the acrylamide gel used in preparing k-casein.

Saran wrap packaging film, a product of The Dow Chemical Company, was used to cover the gel during electrophoresis experiments.

Mini-Shaker for stirring small protein samples for gel electrophoresis was obtained from Fisher Chemical Supply Company.

Chemicals

Guanidine·HCl was purchased from the Matheson Company and was recrystallized from a 1:1 (v/v) mixture of absolute methanol and ethyl ether as described by Greenstein and Jenrette (1942). Urea was obtained from Mallinckrodt and was recrystallized from 60% ethanol and

dried below 60° C. in a vacuum oven. 2-mercaptoethanol (2-ME) and N, N, N, N-tetramethylethylenediamine (TAME) was obtained from Eastman Organic Chemical Products. The dithiothreitol (Cleland's reagent) was obtained from Calbiochem. Crystalline Rennin (60,000 rennin units/g) and N-acetylneuraminic acid were obtained from General Biochemicals. The hydrolyzed starch for starch-urea electrophoresis was purchased from Connaught Medical Research Laboratories. The Cyanogum No. 41 used in polyacrylamide gel electrophoresis (PAGE) was ordered from the American Cyanamide Company. Ammonium persulfate was supplied by Baker Chemical Company. Bio-Gel P-2 and P-20, used in column chromatography, was obtained from Bio-Rad Laboratories. The antiobiotics, terramycin and aureomycin, were gifts from the Michigan State University Swine Research Department. Diisopropylfluorophosphate (DFP) was purchased from the Aldrich Chemical Company.

All other organic or inorganic reagents employed were of reagent grade quality.

Procedures

Preparation of Kappa-Casein

By precipitation from skim milk.--Fresh pooled skim milk (1430 ml) was taken as the starting material and ethylenedinitrilotetraacetic acid (EDTA) was added while stirring until the color of the solution changed from

white to yellow. Anhydrous sodium sulfate (25 g/100 ml) was supplied and the solution was stirred for about one hour after which it was centrifuged at 1000G for 20 minutes and the precipitate discarded.

The pH of the supernatant was adjusted to 3.5 with 1 N HCl and again similarly centrifuged to give a solution which was yellow in color and now had a volume of two liters.

Fourteen hundred ml of absolute ethanol was added to the supernanant. Stirring was continued for two minutes and then allowed to stand for 30 minutes, a white precipitate occurred that was removed by gravity filtration.

An additional 0.6 liters of absolute ethanol was added to the filtrate making it approximately 50% (v/v) with respect to ethanol. A granular precipitate formed which was removed by 20 minutes centrifugation at 1000G. Absolute ethanol was continuously added to the supernatant until another precipitate occurred. The isolation scheme for this preparation is shown in Figure 1.

The procedure was repeated on a somewhat smaller scale (900 ml skim milk) and kappa-enriched fractions were obtained from the 50% as well as the 41% ethanolic solutions.

Again, with a smaller volume (900 ml), the preparation was repeated, but the pH was kept above 7.0 during the entire procedure. Following the addition of EDTA, the pH

was adjusted to 7.0 with 1 N HCl. Anhydrous sodium sulfate (250 g/l) was added as before, and the solution centrifuged at 1000g for 20 minutes.

The supernatant was adjusted to pH 8.5 with 1 N NaOH and made 41% (v/v) with respect to ethanol by the addition of absolute ethanol. The precipitate was removed by centrifugation and was found to be rich in k-casein. The isolation scheme for this reduced scale and modification is outlined in Figure 2.

By preparative horizontal acrylamide gel electrophoresis.--This method was employed for obtaining small amounts of relatively pure k-casein.

Crude k-casein or whole casein was dissolved in 5 M guanidine containing 0.5-1% 2-ME and separated on a horizontal 7% acrylamide preparative gel, using a discontinuous buffer system at pH 8.6 and with a current of 80-100 milliamps (350-400 volts) for 7-8 hours. One 2-cm strip was cut from each side of the gel, stained and replaced in the original gel position. The developed bands were used as a reference to cut out corresponding transverse sections from the central unstained portion of the gel.

The transverse gel sections containing protein were placed in a Waring blender with about 50 ml of distilled water and homogenized for 30-60 seconds. The homogenized gel solution was stirred for several hours at room

temperature; then overnight in the cold (0-5° C.); and again for several hours at room temperature. The solution was centrifuged for 30 minutes at 1000G and the precipitate discarded. The supernatant was filtered, dialyzed against distilled water overnight in the cold (0-5° C.) and lyophilized.

The lyophilized sample was dissolved in water (30 mg/ml) and passed over a Sephadex G-25 column (1.8 cm x 20 cm) using distilled water as the eluting medium. The column elute was monitored for protein absorbance at 280 mu and the first fraction following the void volume was collected and lyophilized. The preparative scheme is outlined in Figure 3.

The lyophilized protein samples were analyzed by SUGE. Results are displayed in Figure 9.

By acid extraction of isoelectric casein.--This method of acid extraction was initiated by Groves (1960) for the isolation of the red protein. Our initial studies of this procedure showed k-casein to be present in the resulting supernatant.

Isoelectric casein was suspended in distilled water to a 3% concentration. The pH was made 3.8-4.0 with dilute acetic acid and the solution stirred with a Lightnin stirrer. For the first several hours, dilute acetic acid was added to maintain the pH at 3.8-4.0 until the pH became relatively constant at room temperature after which the

extract was allowed to stir overnight in the cold (0-5° C.). On the following morning, the pH was adjusted to 4.0 and the mixture was brought up to 40° C. in a water bath whereupon the pH was readjusted to 4.0 and the precipitate was removed by filtration in a Buchner funnel under reduced pressure. Whatman No. 1 filter paper sufficed for this step. The temperature of the mixture at the time of filtering was 35-40° C. The filtrate was clear and colorless.

If the extraction mixture was allowed to separate and the supernatant decanted or exposed to centrifugation to remove the precipitate before filtering, the resulting filtrate was not clear and colorless, but milky colored. The clear, pH 4.0 filtrate was refiltered to remove a few remaining particles and adjusted to pH 4.65; then allowed to stand 24-48 hours in the cold (0-5° C.) or until a precipitate formed. If the filtrate at pH 4.0 was not quite clear precipitation would occur upon adjusting the pH to 4.65 and this could be removed by centrifugation at 1000G for 20 minutes. The resulting supernatant was readjusted carefully to pH 4.65 and held at 0-5° C. until a precipitate occurred which was collected by centrifugation at 1000G for 30 minutes at 0-5° C.

The resulting crude k-casein was recycled several times according to the alcoholic and ammonium sulfate purification procedure of McKenzie and Wake (1961). The preparation scheme is shown in Figure 4.

By acid extraction of isoelectric casein with the addition of sodium chloride.--The procedure is the same as the previous acid extraction method except that 10 g of sodium chloride per liter was added just before adjusting the pH to 3.8-4.0. After adjusting the pH 4.0 filtrate to pH 4.65, it was exhaustively dialyzed against distilled water at 0-5° C. After approximately 36 hours a precipitate formed which was removed by centrifugation in the cold at 1000g for 20 minutes. The precipitate was redissolved in 1 M NaCl at a pH of approximately 6.0 and dialyzed exhaustively against distilled water in the cold (0-5°) until a precipitate occurred, i.e., crude k-casein.

Purification of Kappa-Casein

The purification of crude k-casein preparations was accomplished by utilizing the alcohol-ammonium acetate method of McKenzie and Wake (1961).

Preparation of the Glycomacropeptide

Isoelectric whole casein, three times reprecipitated, was redissolved at pH 6.5 together with 10 mg amounts of antibiotics to prevent bacterial action. Crystalline rennin was mixed with the casein solution and after 15 minutes the enzyme reaction was quenched by making the solution 12% (w/w) with respect to TCA. After filtration, the 12% TCA filtrate was extracted with equal volumes of a 9:1 ethyl ether-N-butanol mixture (v/v) until the pH was about 4.5,

and then the pH was adjusted to 6.5 with 0.1 M NaOH. The filtrate was dialyzed against two changes of 10 volumes of distilled water at room temperature for 4-5 hours; then overnight in the cold (0-5° C.) and lyopholyzed.

Three to ten per cent (w/v) samples of the GMP were run horizontally in 8% polyacrylamide gels (PAG) using: (a) Tris-citrate, borate-NaOH, urea pH 8.6 discontinuous buffer system; and (b) borate-NaOH pH 8.6 continuous buffer system.

Protein staining techniques.--Amido black was employed and the moderately thick acrylamide gels were immersed in the dye bath 2-3 hours before electric destaining in 7% acetic acid. Also submerging the gels for 10 minutes in 95% ethanol and then for 10 minutes in a methanol-water acetic acid mixture (8:10:1 v/v) prior to staining seemed to improve staining of the protein.

Carbohydrate staining techniques.--The techniques employed the method of Keyser (1964). The initial steps in this procedure call for the gels to remain for 10 minutes each in ethanol and in a methanol-water-acetic acid mixture (8:10:1 v/v). This method utilizes periodic acid and basic fuchsin for the staining of carbohydrate containing proteins.

Purification of Glycomacropeptide

Purification of the GMP was achieved by passage through a column (41 cm x 2.8 cm) of Bio-Gel P-2 (50-100 mesh) having a flow rate of 72 ml/hr and a void volume of 64 ml.

The solvent was deionize water. The column effluent was monitored at 280 mμ and absorbance was registered on a recording milliammeter. The GMP was eluted immediately following the void volume and indicative of its molecular size is that Bio-Gel P-2 has an exclusion molecular weight of 3600.

Beta-elimination reaction studies.--These studies, as described by Carubeli, Bhavanandan and Gottschalk (1965), were conducted with the GMP. The samples (0.2 mg/ml) were dissolved in 0.5 N NaOH at room temperature and the increase in absorbance at 241 mμ was observed on the Beckman DU-2 spectrophotometer.

Preparation of Para-Kappa-Casein

P-k-casein was prepared from three main sources:

a. Isoelectric casein
b. P-k-casein produced from calcium (0.25M) treated whole casein by the action of rennin
c. Enriched k-casein

From isoelectric casein.--Isoelectric casein was redissolved under stirring at pH 6.5 by the addition of 1 N NaOH and trace amounts of antibiotics to inhibit bacterial growth. Rennin was added to the solution and the enzyme reaction was halted after 15 minutes by adjusting the pH to 8.5 with 1 N NaOH.

The resulting crude p-k-casein was removed by centrifugation at 1000G for 20 minutes, suspended in distilled water and lyophilized.

From calcium-treated casein.--Calcium-treated whole casein solutions were tested for p-k-casein production. To accomplish this, solid calcium chloride (36.75 g/l) was added slowly and the pH was kept constant at 6.5. The calcium-precipitated casein was removed by centrifugation at 1000g for 20 minutes. P-k-casein was isolated from the supernatant by the same procedure used for isoelectric casein.

The calcium-produced precipitate was suspended in distilled water and stirred vigorously for 15 minutes. The suspended particles were removed by centrifugation at 1000g for 20 minutes followed by gravity filtration. The clear filtrate was treated with rennin and the crude p-k-casein was removed as previously described.

In one case the enzyme reaction contained diisopropylfluorophosphate (DFP) to inhibit possible trypsin action and the reaction mixture was immediately frozen and lyophilized after the reaction had proceeded for 10 minutes.

From crude kappa-casein.--The kappa-enriched fractions obtained by acid extraction of isoelectric casein provided a source of p-k-casein for this study. The kappa-enriched fractions were treated with rennin and the crude p-k-casein was removed by centrifugation as previously described.

This preparation of p-k-casein was purified by disc PAGE before being subjected to ultracentrifugal and chemical analysis.

Purification of Para-Kappa-Casein

The SUGE patterns of crude p-k-casein showed the presence of significant amounts of α- and β-caseins as well as numerous other unknown bands. Several methods were tried in an attempt to obtain relatively pure p-k-casein.

According to solubility differences.--Since p-k-casein is insoluble in most solvent systems it was considered that the other casein components might be removed by proper selection of solvents, pH and temperature.

Veronal buffer pH 8.6 was selected as a solvent and the crude p-k-casein was stirred in this buffer for 48 hours in the cold (0-5° C.).

The object of this operation was to dissolve the absorbed components and leave only the p-k-casein behind as the insoluble matter. The cold temperature was employed because of β-casein's tendency to monomerize and become more soluble at the lower temperatures. The mixture was centrifuged in the cold and the precipitate examined for composition by gel electrophoresis.

Urea solutions (5 M) ranging in pH from 6.0 to 8.6 were tested to selectively solubilize the non-p-k-casein

components. The crude p-k-casein was stirred overnight in the cold and the supernatant removed by centrifugation. The precipitate was checked for purity by SUGE.

A urea solution (5 M), pH 8.6, containing 2-ME was found best to solubilize crude p-k-casein, which was then dialyzed against water and, in other studies, against 1-2 M urea solutions in the cold (0-5° C.). Since the 2-ME aided in solubilizing the p-k-casein, a slow removal of the 2-ME was attempted so that the p-k-casein would become insoluble and the remaining components would still be in solution.

By column chromatography of crude para-kappa-casein.-- This method employed Bio-Gel P-20 (mesh 50-150) which has an exclusion limit of 20,000 molecular weight. The column (1.9 cm x 36 cm) was equilibrated with a 5 M urea solution, pH 8.1, containing 0.1% 2-ME which served also as the eluting solvent. The crude p-k-casein was dissolved in the eluting solvent and passed over the column. The column had a void volume of 49 ml and a flow rate of 30 ml/hr, although at times this flow rate diminished to 10-15 ml/hr. The column was provided with a monitor recording at 280 mμ and fractions were collected based on absorbance peaks.

By preparative disc polyacrylamide gel electrophoresis -- Disc PAGE appeared to be a possible means for purification because of the observation that p-k-casein migrates toward the cathode in gel electrophoresis at pH 8.6. The essential feature of this purification technique utilizes the principle

that all other casein components are allowed to migrate forward into the gel, leaving behind in the supernatant only the p-k-casein.

Five hundred millliliters of 3-5% cyanogum gel were prepared essentially as described by Raymond and Wang (1960) except for the use of N, N, N, N-tetramethylethylenediamine (TAME) instead of dimethylaminopropionitrile. The mold was a glass cylinder 16 cm x 6 cm fitted with a course fritted glass filter at the bottom. After the gel had set up for 20 minutes, excess liquid was poured off and the gel surface carefully rinsed with distilled water. Two-hundred milliliters of the borate buffer (diluted) pH 8.6 was layered on top of the gel surface and the glass column placed in a one-liter beaker. The protein was dissolved in 7 M urea-Tris stock at concentrations up to 5%. With p-k-casein, Cleland's reagent (0.5 mg/ml) was used to insure solubility. A few drops of amido black dye solution was added to provide a method of observing the progress of the electrophoresis. By employing a 5 ml syringe with a 6-inch needle the protein solution was layered on to the surface of the gel and thus also prevented from mixing with the borate buffer which was already in place. The one-liter beaker used as the anode vessel was filled with diluted borate buffer (pH 8.6) to a level of about one centimeter above the gel surface which aided in the dissipation of generated heat.

The anode was placed in the one-liter beaker and the cathode just below the surface of borate buffer atop the

gel. A current of 25 milliamps was applied and electrophoresis was allowed to continue until the dye had moved about 10 cm into the gel. A diagram of the apparatus is shown in Figure 6.

Upon completion, the solution on top of the gel was removed, exhaustively dialyzed and lyopholyzed.

Rennin Study

The enzyme used to prepare p-k-casein in this study was crystalline rennin, Lot 50494, purchased from General Biochemicals, and had an activity of 60,000 rennin units/g. No additional purification steps were employed. The enzyme was analyzed by SUGE, and its sedimentation-velocity coefficient and molecular weight were determined by the ultracentrifuge.

Rennin Action on Alpha and Beta Casein Enriched Preparations

Reaction of rennin with miscellaneous α- and ß-casein-rich fractions.--Rennin was allowed to react with the following casein samples:

a. An alpha-rich sample prepared by the method of McMeekin, Hipp and Groves (1959),
b. An alpha, beta casein-rich fraction from kappa preparation prepared from skim milk (see preparation of k-casein), and
c. An alpha, beta casein-rich precipitate from kappa preparation of Zittle (1962).

These samples were subjected to rennin action for ten minutes. The reaction mixture was frozen, lyopholyzed and analyzed on SUGE.

Reaction of rennin with calcium precipitated casein.--The precipitate which remained after acid extraction of casein (pH 4.0) was dissolved in water at pH 6.5. The solution was made 0.25 M with respect to calcium ions, with the pH kept constant at 6.5. The precipitate was removed by centrifugation at 1000g for 20 minutes. DFP was added to the supernatant to inhibit any trypsin and then the mixture was treated with rennin. The addition of DFP decreased the pH, necessitating an adjustment to 6.0 with 0.1 N NaOH. After ten minutes the reaction was terminated by freezing and lyopholyzation. The sample was run in duplicates on horizontal PAGE, staining one-half of the gel for protein and assaying for calcium-sensitive protein bands on the other half of the gel. The calcium sensitivity of the resolved proteins was determined by placing the gel in an 0.25 M calcium chloride solution as described by McCabe and Brunner (1966).

Horizontal Gel Electrophoresis

Starch-urea gel (SUG).--The procedure employed is essentially those described by Wake and Baldwin (1961) and Smithies (1955). After pouring the gels into the horizontal Plexiglass cells they were allowed to remain in a deep, covered pan until the gel had hardened. The gels

were then covered with Saran wrap to prevent dehydration.

Prior to applying the protein samples, the gel was subjected to the operating current for about one hour. After the samples were introduced into the slots of the gel, a current of 35-40 milliamps (140 volts) was applied. The experiment was terminated when the migrating front had traveled approximately 10 cm beyond the slot position.

Since there was a need to check the cathode migrating components the slots were placed usually 6-7 cm from the cathode end of the gel. Placing the slots this far from the cathode end of the gel increased the time required for the migrating front to move 10 cm beyond the sample origin. The usual running time for a gel of this type was about 20 hours.

The gels could be operated at room temperature since the Plexiglas cells were constructed with compartments on the underside through which cold tap water ran to keep the gel from over-heating.

Polyacrylamide gel (PAG).--The polyacrylamide gels were prepared according to the procedures described by Peterson (1963) and Raymond and Wang (1960). The Plexiglas cells were constructed such as to allow direct buffer-gel contact and no filter paper between gel and buffer was necessary. The construction also provided a cooling space

on the underside of the gel bed similar to that described in SUG. The voltage and current used in PAGE was the same as employed in SUGE. The operating current was applied for one hour prior to addition of samples.

Preparative horizontal polyacrylamide gel.--The only difference between the preparative gel and PAG was the size of the Plexiglass cell. The preparative cell was 30 cm x 36 cm and contained a built-in cooling system, as previously described, and contained one long, continuous, sample slot. The 1-2 ml samples (3-5% protein) were introduced with a syringe and the operating current was maintained at 80-100 milliamps for 7-8 hours.

Upon completion of the run 2-cm strips were cut lengthwise from the PAG parallel to direction of migration, from the left and right sides of the gel. These strips were stained with amido black and destained electrically. They were then replaced in their original positions in the gel and their bands were used as markers in cutting out corresponding unmarked band areas from the original gel.

Buffers for gel electrophoresis.--

Tris stock = 0.76 M pH 8.6
91.96 g Tris and 12.05 g citric acid diluted to 1 liter

Borate-NaOH Buffer = pH 8.65
881 g boric acid and 190 g NaOH diluted to 19 l

When used in the electrode vessels, two volumes of the borate-NaOH buffer was diluted with three volumes of water

Discontinuous buffer system.--The discontinuous buffer system employed Tris stock buffer in the gel preparation, usually 10% of the final gel volume and the electrode vessels contained diluted borate-NaOH buffer.

Continuous buffer system.--In this case the diluted borate-NaOH was substituted for Tris stock in the preparation of the gel. The electrode vessels contained diluted borate-NaOH buffer.

Sample buffers.--The protein was dissolved in a Tris stock buffer containing 5 M guanidine pH 8.6. When necessary a disulfide cleaving agent was added to the sample buffer just prior to dissolving the sample. The protein samples were weighed out and dissolved in a one dram screw cap vial and stirred for 30 seconds on a Mini-Shaker or until all protein was dissolved. All samples were then allowed to stand for 15 minutes before being applied to the gel to insure complete dissociation.

Staining.--Protein staining in the gels after electrophophoresis employed amido black as the binding dye. SUG were immersed in the dye solution for 15 minutes before electrolytic destaining. PAG usually remained in the amido black solution for one hour before destaining.

Carbohydrate staining of the PAG after electrophoresis involved the method of Keyser (1964). The gels were operated so that each sample was run in duplicate and upon completion of the electrophoretic experiment the gel was cut lengthwise in half. One-half was stained for carbohydrate and due to the extensive length of the time in alcoholic solutions required for the carbohydrate staining and destaining techniques, this half of the gel had a tendency to shrink to about 70% of its original size.

Physical Studies

Sedimentation-velocity.--Analysis were performed at 20° C. using a synthetic boundary cell and a rotor speed of 59,730 RPM. The cell was filled by placing 0.350 ml of protein solution in the lower compartment and 0.1 ml of solvent in the upper compartment. Sedimentation coefficients were determined by plotting the logarithm of the maximum ordinate against time.

Diffusion constants.--These experiments employed a double-sector synthetic boundary cell which contained 0.25 ml of protein solution and 0.35 ml of solvent. The protein boundary formed at a low rotor speed of 4000 RPM. At this speed the boundary does not move appreciably and thus any boundary spreading due to heterogeneity is minimized. The diffusion constants were evaluated by plotting the square of the second moment against time as described by Schachman (1957).

Molecular weight determinations.--The molecular weights were arrived at by two methods. First, the short column procedure was adapted as described by Van Holde and Baldwin (1958) whereby the centrifugations were performed in a double-sector synthetic-boundary cells using a solution column length of 1-2 mm. This method provides an apparent weight-average molecular weight (M^{app}) for the entire cell contents. The true molecular weight (Mw) is obtained by extrapolation of M^{app} to zero concentration.

A z-average molecular weight (Mz) can be obtained utilizing information derived from the meniscus and cell bottom. The Mz determination is useful in polydisperse systems and the calculation is more sensitive to the higher molecular weight species. In a monodisperse system, where all molecular species are the same, the Mz and M^{app} would be the same. In a polydisperse system the Mz would be greater than the M^{app}. The evidence for polydispersion is usually expressed by the Mz/M^{app} ratio. In a monodisperse system this ratio would be one, but in a polydisperse system this ratio would be greater than one.

Secondly, the approach-to-equilibrium method was used to determine the low molecular weight component in a heterogeneous system. The experiments were performed at various rotor speeds and the data plotted as described by Trautman (1956) and adapted to heterogeneous systems according to the procedure of Erlander and Foster (1959).

Densities of solvents.--Determinations were made with a 25 ml pycnometer at 20° C. and solution densities were calculated according to Fujita (1962) using the relationship:

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

where $\bar{v}$ is the partial specific volume of the protein and C its concentration in g/ml. Freshly boiled, redistilled water was used to calibrate the pycnometers.

Partial specific volume.--This volume was calculated from the amino acid analysis of Jolles et al. (1962) using the relationship:

$$\bar{v} = \frac{\Sigma_i \bar{v}_i W_i}{\Sigma_i W_i}$$

where $\bar{v}$ is the partial specific volume of the protein, $\bar{v}_i$ is the partial specific volume of the i^{th} amino acid residue and W_i is the weight per cent of the i^{th} amino acid residue (Cohn and Edsall, 1943; and McMeekin, Groves and Hipp, 1949). The weight per cent of the amino acid residue is given by:

$$W - g_i/100 \text{ g protein } (1 - \frac{18}{M_i})$$

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

Free-boundary electrophoresis.--The electrophoretic analysis was performed in a two ml cell assembly, using 0.1 M veronal buffer pH 8.6 and a temperature of 2° C.

Chemical Composition

Ultra violet spectrum.--A 0.3 mg/ml concentration of GMP in 0.1 N NaCl (pH 6.0) was scanned in a recording spectrophotometer from 200 mu to 340 mu at room temperature.

Amino acid analysis.--About 2 mg of protein was hydrolyzed in 6 N hydrochloric acid in a sealed, evacuated glass tube. Hydrolysis of each protein was done in duplicate for the time periods of 20 and 70 hours, at 110° C. in a forced draft air laboratory oven.

Both the standard calibration runs and the protein hydrolysate runs contained 0.1 micromoles of norleucine as an internal standard.

Phosphorous.--was determined colorimetrically in a wet digest (H_2SO_4 and H_2O_2) of the protein by the method of Sumner (1944).

Hexose.--determinations employed the method of Dubois, Gilles, Hamilton, Rebers and Smith (1956) using a 5% phenol solution and a 1:1 (w/w) mixture of galactose and mannose as a standard.

Hexosamine.--determinations utilized the method of Cessi and Philliego (1960) as modified by Johansen, Marshall and Neuberger (1960). The samples were hydrolyzed for six hours in a 4 N HCl at 100° C. and glucosamine·HCl was used as the standard.

Sialic acid.--content determinations employed the thiobarbituric acid assay by Warren (1959). N-acetylneuraminic acid previously dried under vacuum and P_2O_5 was used as the standard.

Tryptophan assays.--followed procedure B as described by Spies and Chambers (1948) with incorporated modifications.

TABLE 1.--Physical analysis of kappa-casein.

Conc. mg/ml	Analytical Ultracentrifuge Data		
	Solvent System	$S_{20,w} \times 10^{13}$	Mole. wt.
4.0[a]	4 M guanidine pH 6.4	2.1	
5.0[a]	5 M guanidine + 0.01% 2-ME pH 6.1	1.7	
9.0[b]	phosphate buffer $\Gamma/2 = 0.1$ pH 7.1	13.9	
10.0[a]	5 M guanidine + 0.01% 2-ME pH 8.1		19,022

[a]Horizontal preparative PAUGE preparation.

[b]Acid extraction of isoelectric casein preparation.

TABLE 2.--Physical analysis of the glycomacropeptide.

Analytical Ultracentrifuge Data			
Conc. mg/ml	Mole. wt.[a]	$S_{20,w} \times 10^{13}$	$D_{20,w} \times 10^{7}$
7.2	7459	0.997	26.8
6.1	7615	1.029	26.2
5.0	7415	1.063	26.7
0.0	7500[b]	1.2[b]	26.4[b]

Electrophoretic Mobility in Veronal Buffer		
pH = 8.6	r2 = 0.1	Protein conc. = 2%
Ascending Pattern -5.51 T.U.		Descending Pattern -4.95 T.U.

[a] Sedimentation equilibrium.

[b] Extrapolated values.

TABLE 3.--Chemical analysis of the glycomacropeptide.

Component	This Study	Brunner and Thompson (1959)	Jollés and Alais (1960)
	%	%	%
Phosphorous	0.72	0.63	0.37
Hexose	4.34	5.1	7.4
Hexose[a]	2.36	---	---
Hexosamine	3.89	2.3	6.5
Sialic Acid	8.15	11.3	14.3
Tryptophan	0.05	---	---

[a]After alkaline hydrolysis.

TABLE 4.--The amino acid composition of glycomacropeptide.

Residue	Number of Residues			
	This Study[a]	Jollés et al. (1963)[c]	Kalan & Woychik (1965)[c]	DeKoning et al. (1966)[b,c]
Asp	4	3	3	4
Thr	10	7	7	9
Ser	6	4	3	5
Glu	9	8	9	9
Pro	7	6	5	7
Gly	1	1	1	1
Ala	5	4	4	5
Cys'	-	-	-	-
Val	5	4	4	5
Met	1	-	1	1
Ile	5	5	5	5
Leu	1	1	1	1
Tyr	-	-	-	-
Phe	-	-	-	-
Lys	3	3	2-3	3
His	-	-	-	-
Arg	-	-	-	-
Trp	-	-	-	-
NH_3	-	-	-	13.3

[a]Average of duplicate samples for each time.

[b]Average for both genetic variants.

[c]Based on molecular weight of 8,000.

TABLE 5.--Physical characteristics of para-kappa-casein.

Ultracentrifuge Analysis				
Conc. mg/ml	Mole. wt. equilibrium	Mole. wt. Trautman plot	$S_{20,w}$	$D_{20,w}$
2.5	14,200			
4.2	14,550			
5.1	14,600			11.3
6.1	14,500	13,636	1.12	11.6
7.5	15,200		1.04	
10.1		13,504	1.02	
0.0	14,000[a]		1.25[a]	

Molecular weights from diffusion and sedimentation data:

5.1 mg/ml - 14,600 6.1 mg/ml - 14,200

$\bar{v}$ = 0.734

[a]Extrapolated values.

TABLE 6.--Chemical analysis of para-kappa-casein.

Component	Compositional Analysis %
Phosphorous	0.02
Hexose	2.30
Hexosamine	0.06
Sialic Acid	trace
Tryptophan	0.05

TABLE 7.--The amino acid composition of para-kappa-casein.

Residue	Number of Residues			
	This Study[a]	Jollés et al. (1963)[b]	Kalan & Woychik (1965)[c]	DeKoning et al. (1966)[b]
Asp	6	13	12	11
Thr	3	7	12	6
Ser	6	13	11	11
Glu	15	28 ± 1	26	27
Pro	10	17	17	18
Gly	1	4	3	3
Ala	8	13 ± 1	13	14
Cys'	-	1-2	2	2
Val	5	10	10	9
Met	1	1-2	2	2
Ile	5	10 ± 1	11	9
Leu	7	12 ± 1	9	11
Tyr	7	13 ± 1	9	13
Phe	3	6	4	6
Lys	7	12	6	9
His	3	4	3	4
Arg	3	6 ± 1	5	7
Trp	-	2	-	2
NH_3	-	-	-	25

[a]Based on internal standard of norleucine.

[b]Based on molecular weight of 20,000.

[c]Based on a ratio of eight amino acids.

TABLE 8.--Adjusted amino acid composition of para-kappa-casein.

Residue	Number of Residues		
	This Study	Jollés et al. (1963)[a]	DeKoning et al. (1966)[a]
Asp	6	7	7
Thr	3	4	2
Ser	6	7	7
Glu	15	16	16
Pro	10	10	11
Gly	1	2	2
Ala	8	7	8
Cys'	-	1	1
Val	5	6	5
Met	1	1	1
Ile	5	6	5
Leu	7	7	7
Tyr	7	7	8
Phe	3	3	4
Lys	7	7	5
His	3	2	2
Arg	3	3	4
Trp	-	1	1
NH_3	-	-	15

[a]Adapted from original and calculated on basis of 11,634 as molecular weight of para-kappa-casein.

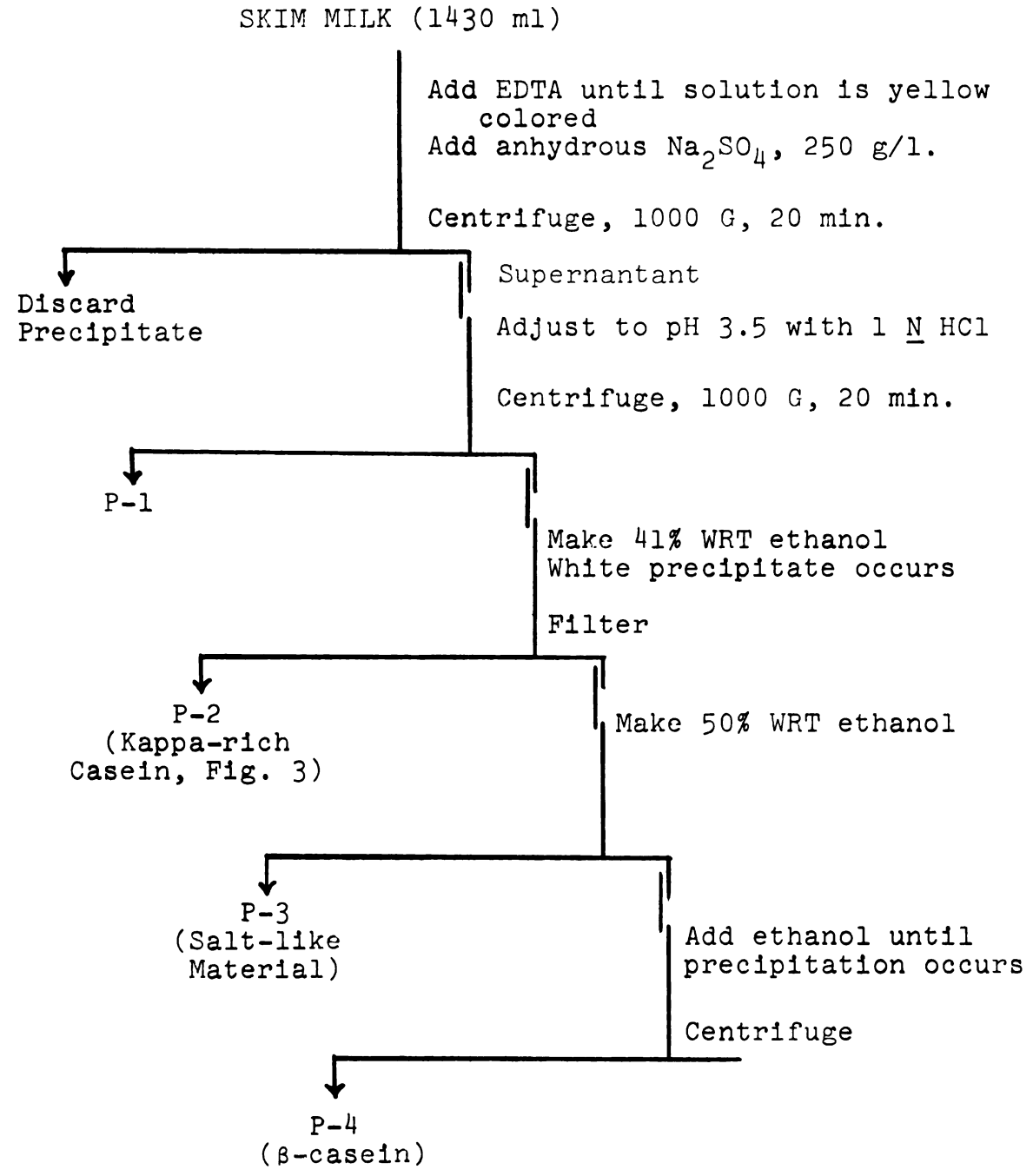


Figure 1.--Kappa-casein preparation from skim milk.

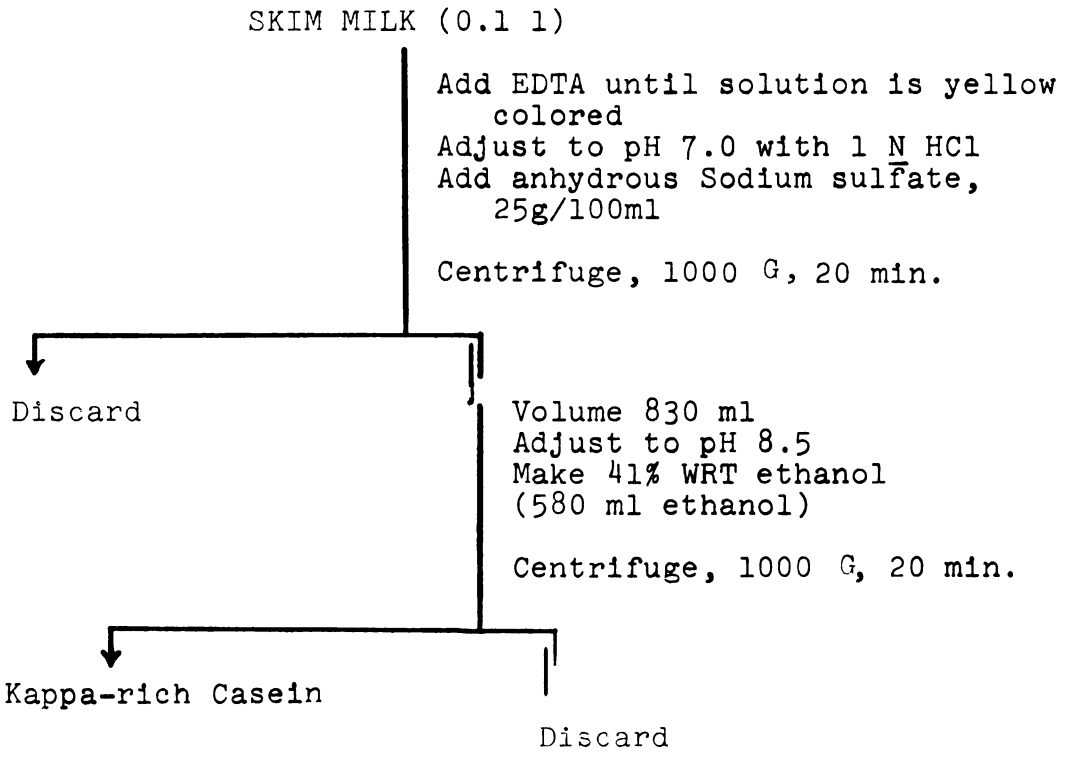


Figure 2.--Kappa casein preparation from skim milk.

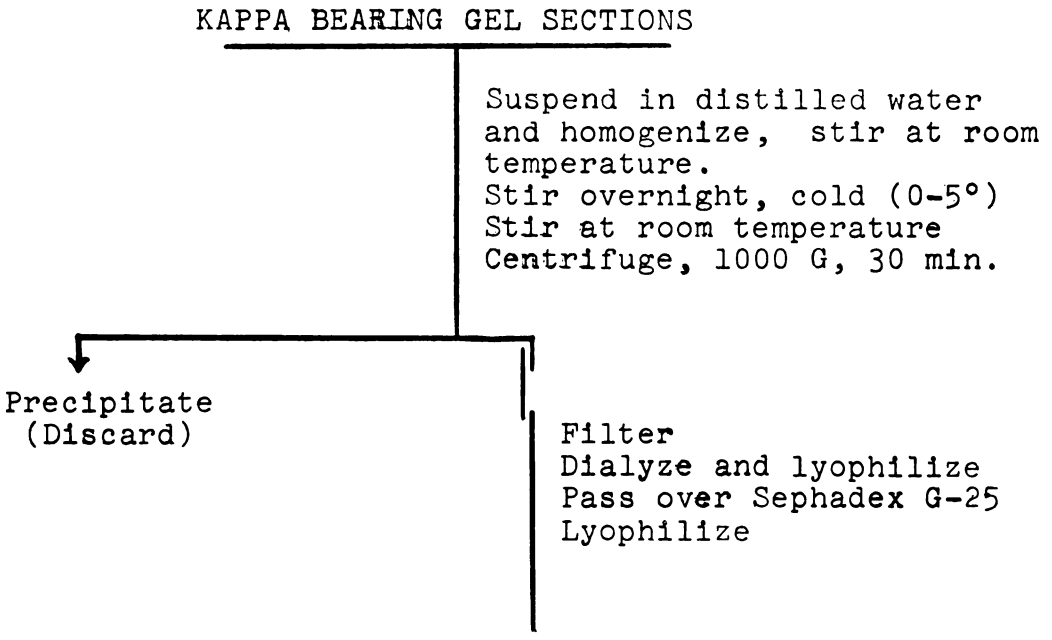


Figure 3.--Horizontal polyacrylamide urea gel preparation of kappa-casein.

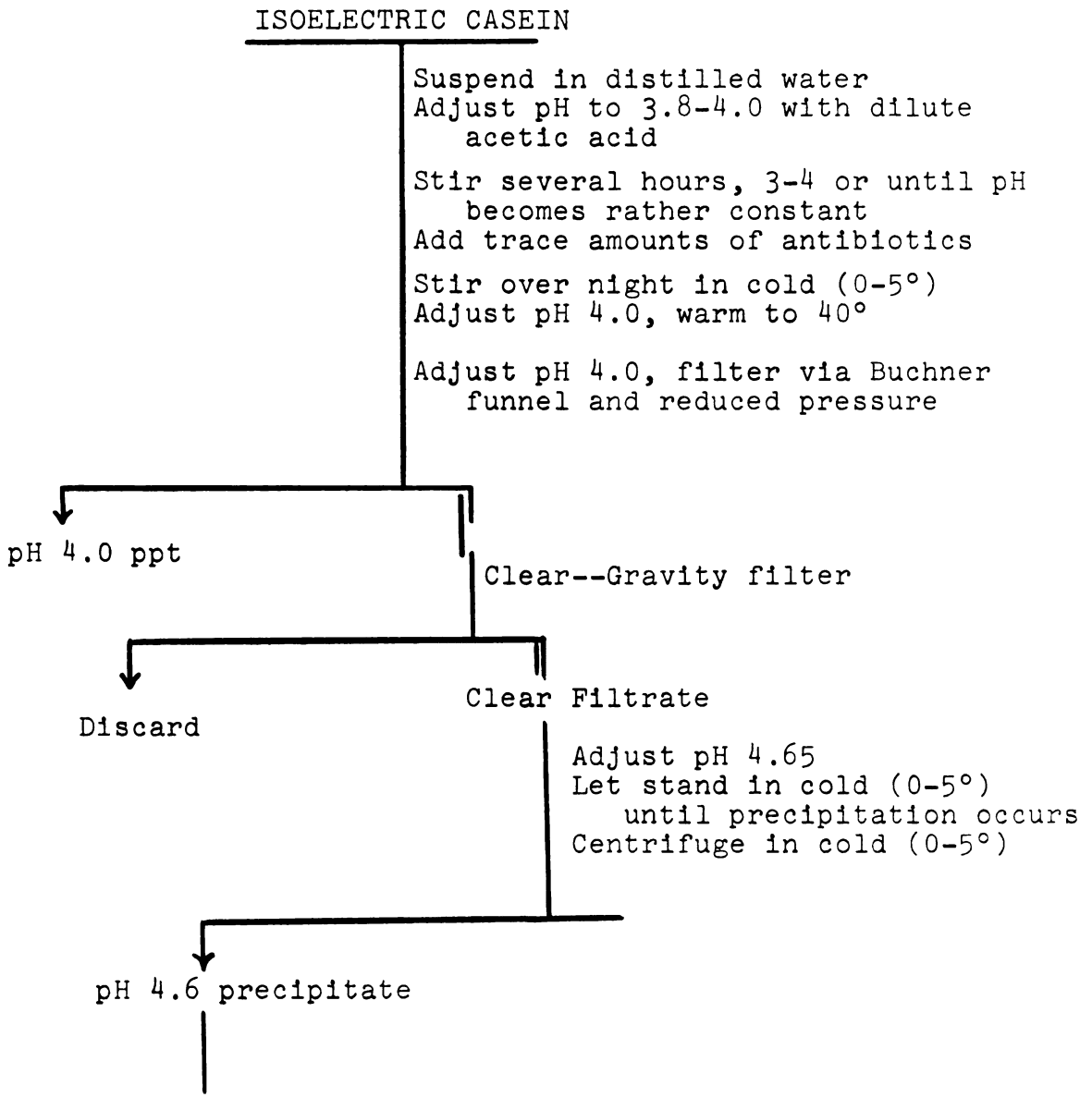


Figure 4.--Acid extraction method for the precipitation of crude kappa-casein.

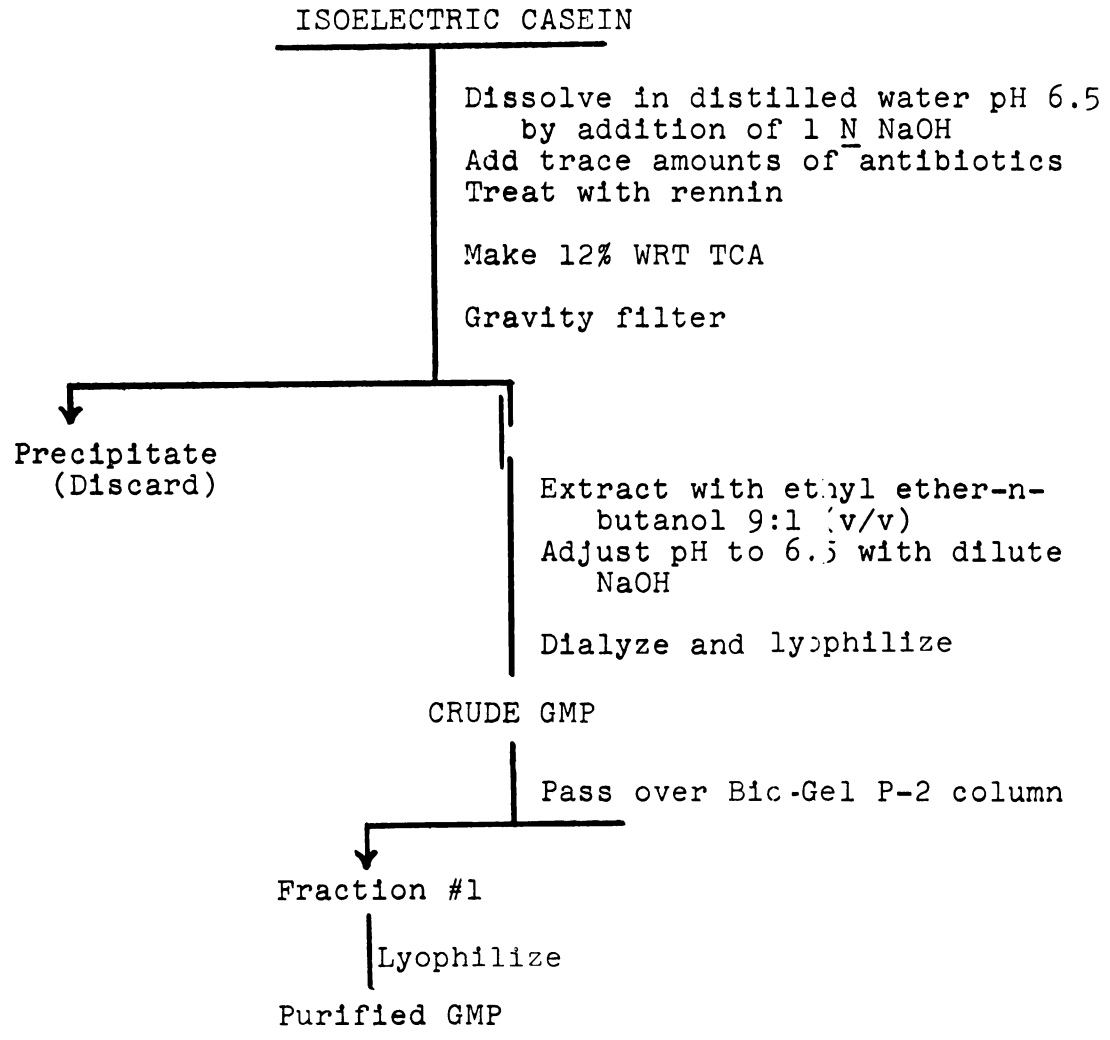


Figure 5.--Preparation scheme for Glycomacropeptide (GMP)

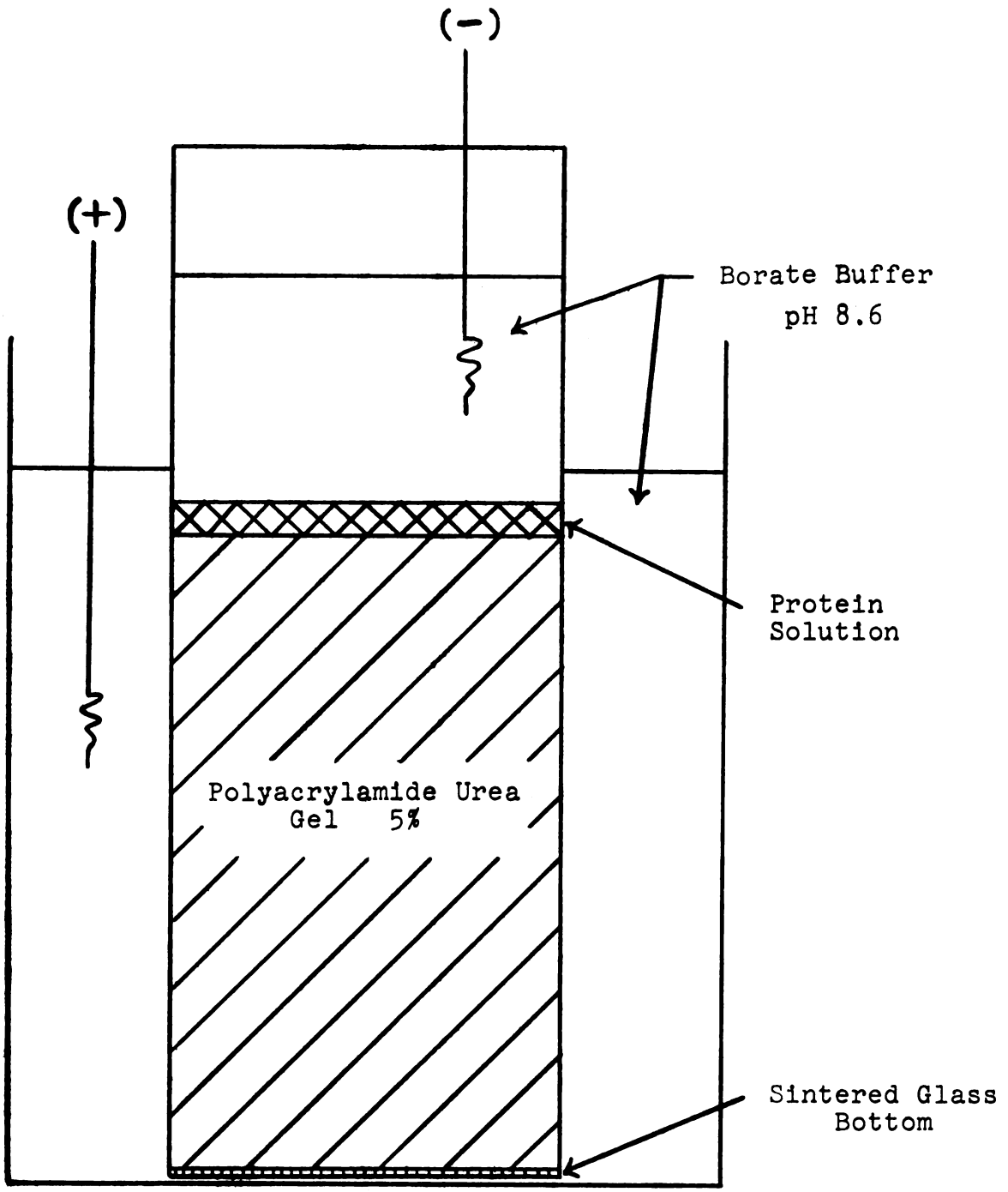


Figure 6.--Diagram of preparative disc polyacrylamide gel apparatus used in purification of para-kappa-casein.

Figure 7.--Starch-Urea Gel Electrophoretograms of Kappa-Casein Preparations from Skim Milk.

Slot A - Precipitate obtained from skim milk--EDTA preparation at pH 3.5 and 41% ethanol.

Slot B - Precipitate obtained from modified skim milk--EDTA preparation at pH 8.5 and 41% ethanol.

Slot C - Precipitate obtained from skim milk (900 ml) --EDTA preparation at pH 3.5 and 41% ethanol.

Slot D - Precipitate obtained from skim milk (900 ml) --EDTA preparation at pH 3.5 and 50% ethanol.

Figure 8.--Preparative Horizontal Polyacrylamide Gel Electrophoretogram.

A longitudinal strip from preparative horizontal PAUGE and stained to show the location of k-casein bands.

Figure 9.--Starch-Urea Gel Electrophoretograms of Kappa-Casein Excised from Horizontal Preparative Polyacrylamide Gels.

Slot A - k-bands 2, 3 and 4 (see Figure 8) excised as a group.

Slot B - k-bands 1, 2, 3 and 4 (see Figure 8) excised as a group.

Slot C - k-band 2.

Slot D - k-band 3.

Slot E - k-band 4.

Slot F - Crude k-casein used to compare the positions of the isolated k-bands with those of α- and β-casein.

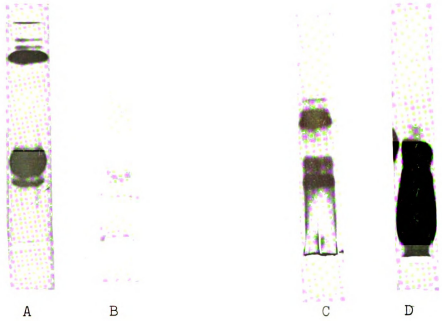


Figure 7

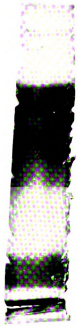


Figure 8

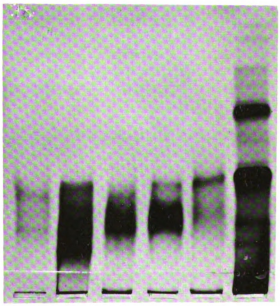


Figure 9

Figure 10.--Electrophoretograms of Kappa-Casein Prepared from Acid Extraction of Isoelectric Casein.

Slot A - SUGE of k-casein-rich precipitate obtained after pH 4.0 filtrate was adjusted to pH 4.6

Slot B - SUGE of k-casein after purification of the material represented in Slot A by the alcohol ammonium sulphate procedure of McKenzie and Wake (1961).

Slot C - PAGE of k-casein material represented in Slot B with Cleland's reagent used to reduce any disulfides in the k-casein.

Figure 11.--Starch-Urea Gel Patterns Showing Acid Extracted Kappa-Casein, the Effect of Rennin on Such Kappa-Casein and Purified Para-Kappa-Casein.

Slot A - Acid extracted k-casein preparation before the addition of rennin.

Slot B - Acid extracted k-casein preparation (Slot A) after the addition of rennin. (Note presence of one major p-k-casein band.)

Slot C - K-casein after purification of the material represented by Slot B. (Note the appearance of two major p-k-casein bands.)

Figure 12.--Polyacrylamide Urea Gel (+2-Mercaptoethanol) of Acid Extracted Kappa-Casein, Amido Black and Periodic Acid--Schiff Base Developed.

Slot A - Acid extracted k-casein stained for protein content with amido black.

Slot B - Acid extracted k-casein stained for glycoprotein content with PAS. The PAS stain appeared in the area within the indicated circle and not in one specific band or region.

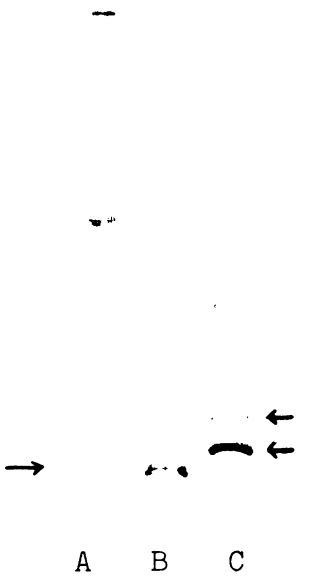
Figure 13.--Starch-Urea Gel (+2-Mercaptoethanol) Electrophoretograms Showing the Effect of Sodium Chloride on Kappa-Casein.

Slot A - Acid extracted k-casein (10 g/l NaCl) after dialyzing out sodium chloride.

Slot B - Material represented in Slot A after redissolving in NaCl and redialyzing.

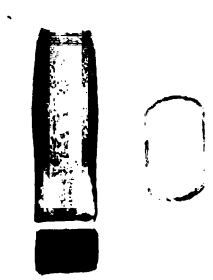
A B C

Figure 10



A B C

Figure 11



A

B

Figure 12

A

B

Figure 13

Fiugre 14.--Discontinuous Buffer--Polyacrylamide Gel Electrophoretograms of Glycomacropeptide, Amido Black and Periodic Acid--Schiff Base Developed.

Slot A - Crude GMP stained with amido black for protein. Arrow indicates position of GMP.

Slot B - Purified GMP stained with amido black for protein. Arrow indicates position of GMP.

Slot C - Crude GMP stained with amido black for protein. Arrow indicates position of GMP (very faint).

Slot D - Miscellaneous caseins stained with amido black.

Slot E - Miscellaneous caseins stained with amido black.

Slot F - Crude GMP stained with PAS. Arrow indicates Position of GMP.

Slot G - Miscellaneous caseins stained with PAS.

Slot H - Miscellaneous caseins stained with PAS.

Slot I - Crude GMP of Slot B counterstained with amido black.

Slot J - Miscellaneous caseins counterstained with PAS.

Slot K - Miscellaneous caseins counter-stained with PAS.

NOTE: Counterstaining of Slots F, G and H with amido black yielded Slots I, J and K which is similar to original amido black stain as shown in Slots C, D and E.

Figure 15.--Continuous Buffer--Polyacrylamide Gel Electrophoretograms of Glycomacropeptide, Amido Black and Periodic Acid--Schiff Base Developed.

Slot A - Crude GMP stained with amido black. Arrows indicate location of bands.

Slot B - Crude GMP stained with PAS. Arrows indicate location of bands.

Figure 16.--Continuous Buffer--Polyacrylamide Gel Electrophoretograms of Purified Glycomacropeptide, Amido Black and Periodic Acid--Schiff Base Developed.

Slot A - Purified GMP stained with amido black. No protein bands were present.

Slot B - Purified GMP stained with PAS. Arrow indicates position of band.

Slot C - Slot B after counterstaining with amido black. Arrow indicates position of band.

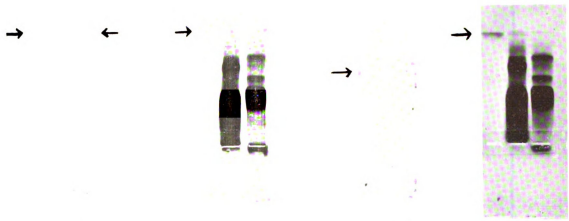


Figure 14

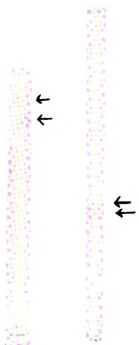


Figure 15

Figure 16

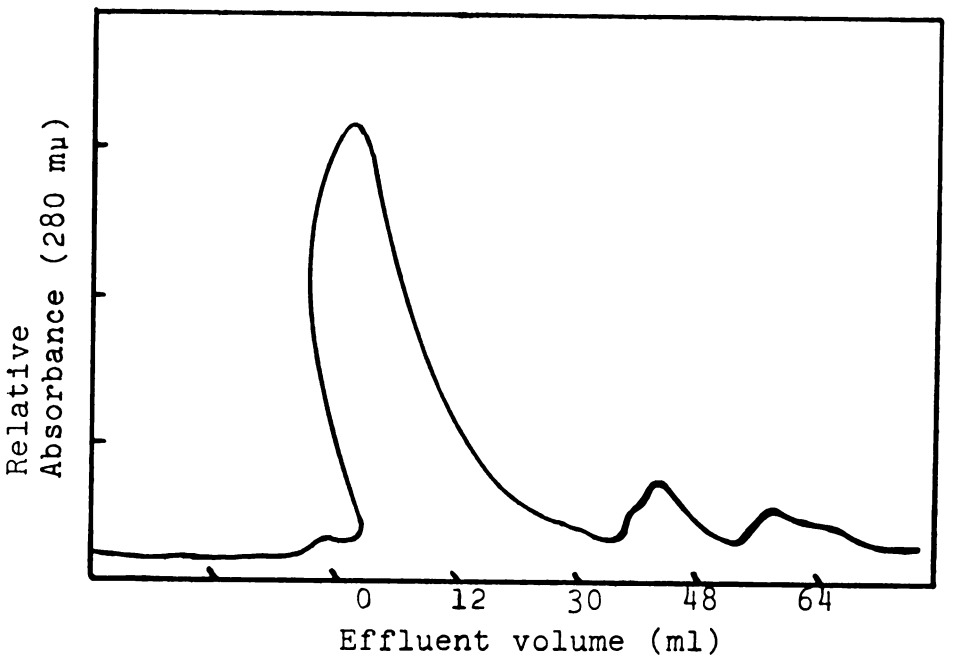


Figure 17.--Chromatographic profile of 12% trichloroacetic acid soluble fraction over Bio-Gel P-2.

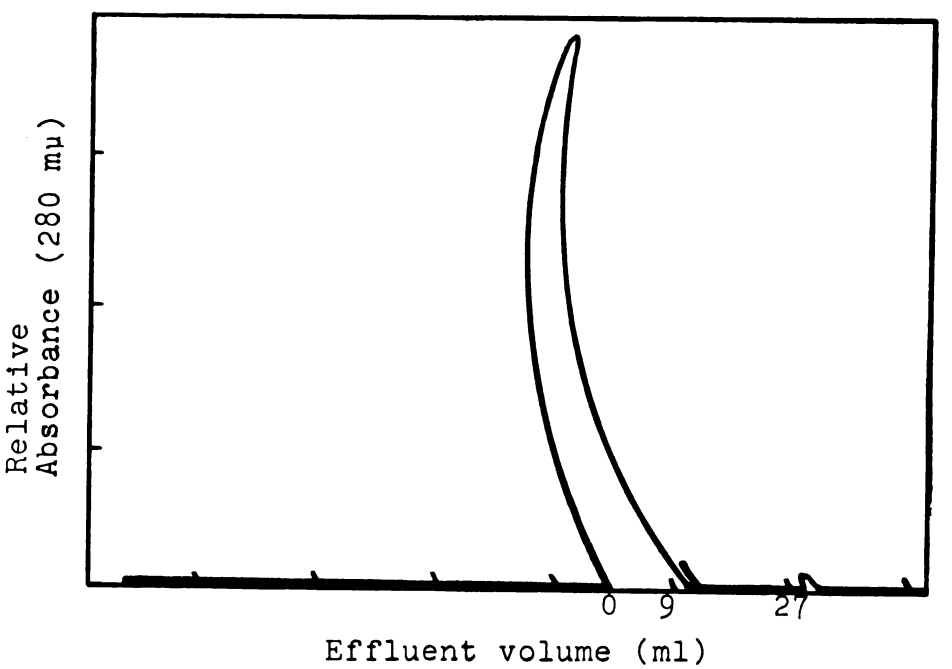


Figure 18.--Chromatographic profile of purified glycomacropeptide on Bio-Gel P-2 following alkaline hydrolysis.

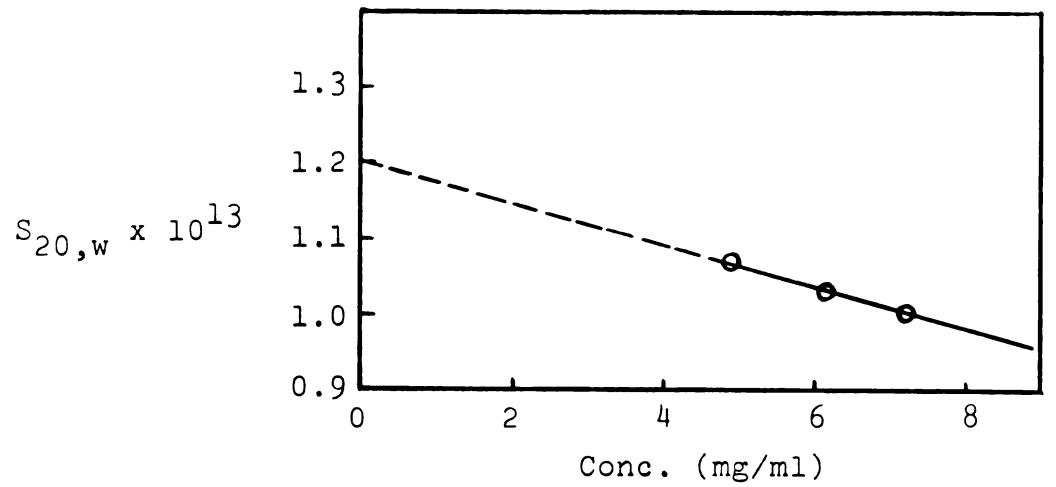


Figure 19.--Plot of sedimentation-velocity coefficient versus concentration for glycomacropeptide.

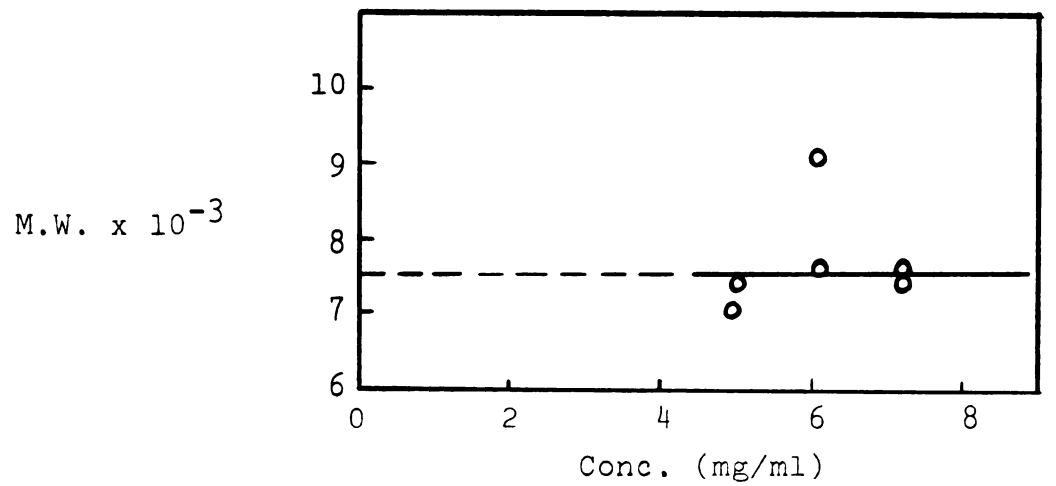


Figure 20.--Plot of apparent molecular weight versus concentration for glycomacropeptide.

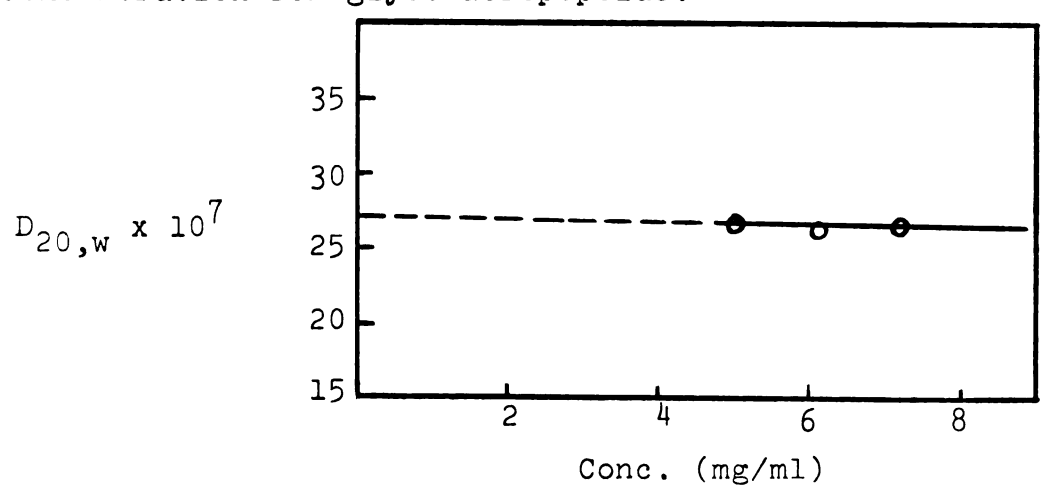


Figure 21.--Plot of apparent diffusion constants versus concentration for glycomacropeptide.

Figure 22.--Schlieren Patterns for the Glycomacropeptide and Para-Kappa-Casein.

A - GMP 6.1 mg/ml 0.1 M NaCl pH 6.0
Sedimentation-velocity--59,780 RPM T = 20° t = 30 min

B - GMP 6.1 mg/ml 0.1 M NaCl pH 6.0
Sedimentation equilibrium--24,630 RPM T = 20° θ = 60°

C - P-k-casein abnormal initial concentration pattern.

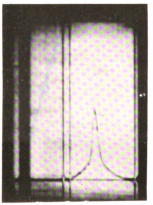
D - P-k-casein 6.1 mg/ml 5 M guanidine + Cleland's reagent pH 8.1
Normal initial concentration pattern.

E - P-k-casein 6.1 mg/ml 5 M guanidine + Cleland's reagent pH 8.1
Sedimentation-velocity--59,780 RPM T = 20° t = 64 min

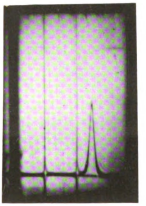
F - P-k-casein 6.1 mg/ml 5 M guanidine + Cleland's reagent pH 8.1
Sedimentation equilibrium--20,410 RPM T = 20° θ = 70°

A

B



C



D

E

F

Figure 22

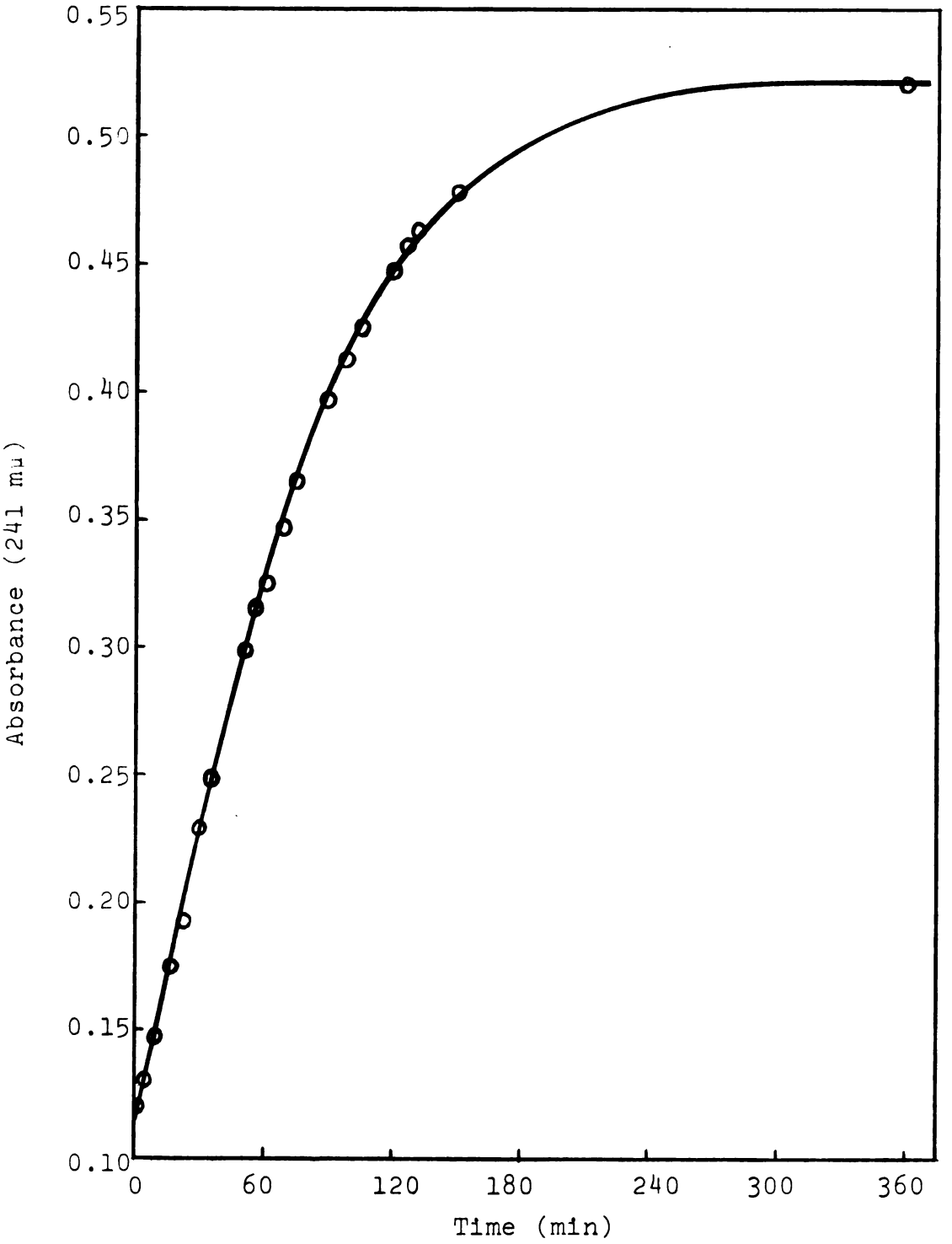


Figure 23.--Plot of absorbance versus time for alkaline hydrolysis of glycomacropeptide.

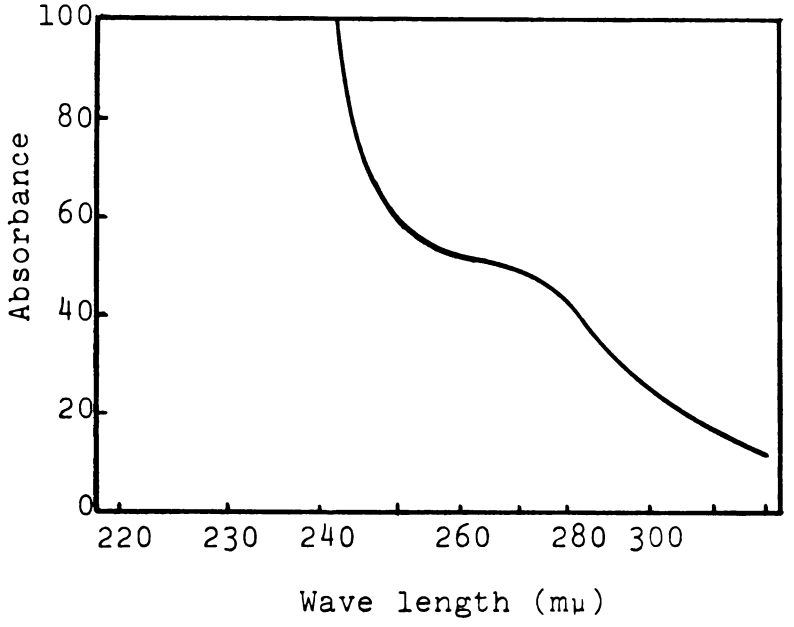


Figure 24.--Absorption Spectrum of Glycomacropeptide.

Figure 25.--Starch-Urea Gel Electrophoretograms of Para-Kappa-Casein Made with Rennin from Isoelectric Casein.

Slots A and B - Crude p-k-casein prepared from isoelectric casein.

Slot C - Material represented by Slots A and B after purification based on solubility differences in a veronal 5 M urea-2-ME pH 8.6 solution.

Figure 26.--Starch-Urea Gel Electrophoretograms of Para-Kappa-Casein Made with Rennin from Calcium-Treated Isoelectric Casein.

Slot A - Crude p-k-casein resulting from the addition of rennin to the supernatant of 0.25 M treated isoelectric casein.

Slot B - P-k-casein after purification of the material represented in Slot A by preparative disc PAUGE. (Note the appearance of two new major p-k-casein bands in Slot B.)

Figure 27.--Starch-Urea Gel Electrophoretograms of Crude Para-Kappa-Casein Passed Through a Bio-Gel P-2 Column. The column was equilibrated with 5 M urea-2-ME prior to addition of the protein.

Slot A - Crude p-k-casein, starting material.

Slot B - Fraction 1 from peak 1.

Slot C - Fraction 2 from peak 2.

Slot D - Fraction 3 from peak 3.

Slot E - Fraction 4 from peak 4.

Slot F - Fraction 5 from peak 5.

NOTE the change in the number of p-k-casein bands and the difference in migration as fractionation continues.

Figure 28.--Starch-Urea Gel Electrophoretograms of Purified Para-Kappa-Casein.

Slots A and B - Two preparations of p-k-casein prepared by treatment of rennin on enriched k-casein obtained by acid extracted-method and purified by preparative disc PAUGE.

Figure 29.--Starch-Urea Gel Electrophoretograms of Eight Different Para-Kappa-Casein Preparations.

Slots A-H contain eight different p-k-casein preparations in varying degrees of purity. (Note the number of cathodic bands and the great distance that some of them had migrated.)

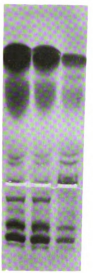


Figure 25

Figure 26

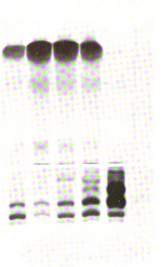


Figure 27

Figure 28

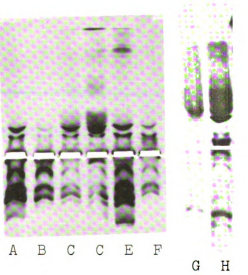


Figure 29

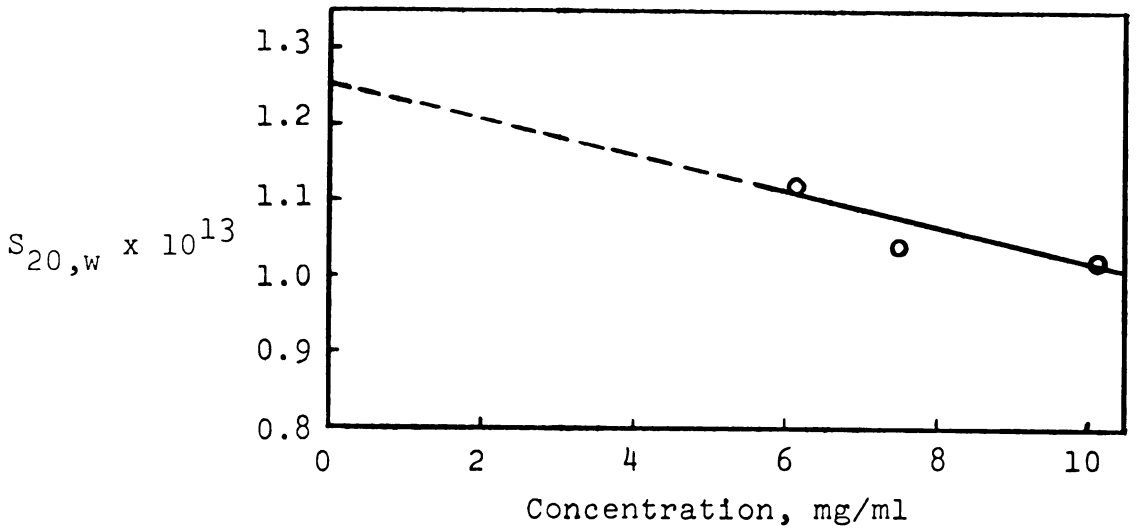


Figure 30.--Plot of sedimentation-velocity coefficient versus concentration for para-kappa-casein.

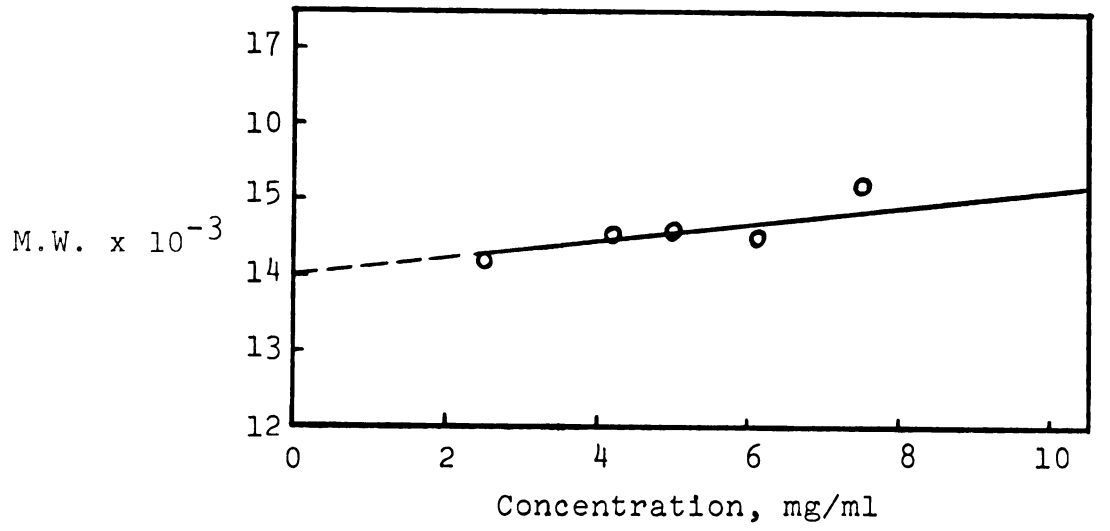


Figure 31.--Plot of apparent molecular weight versus concentration for para-kappa casein.

Figure 32.--Starch-Urea Gel (+2-Mercaptoethanol) Electrophoretograms of Crystalline Rennin.

Figure 33.--Polyacrylamide Urea Gel Electrophoretograms Showing the Effect of Rennin on Alpha- and Beta-Casein-Rich Fractions.

Slot A - PAUGE (+2-ME) of precipitate from 0.25 M calcium-treated isoelectric casein. The precipitate was extracted with water, filtered and lyopholyzed.

Slot B - PAUGE (+2-ME) of the material in Slot A after treatment with rennin. The arrows indicate the position of newly formed bands.

Figure 34.--Starch-Urea Gel (+2-Mercaptoethanol) Electrophoretograms Showing the Effect of Rennin on Alpha- and Beta-Casein-Rich Fractions.

Slots A, C and E are the starting materials.

Slots B, D and F show the effect of rennin on each starting material.

Figure 32

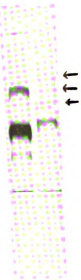


Figure 33

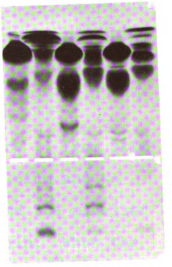


Figure 34

DISCUSSION

Preparation and Properties of Kappa-Casein

Precipitation from Skim Milk

In the preparation of κ-casein (Figure 1) from skim milk, EDTA was added to complex with the calcium and to shift the equilibrium between the soluble and the micellar forms of casein in favor of the soluble casein. When the skim milk attained the yellow color of whey the addition of EDTA was stopped. Anhydrous sodium sulfate was added to salt out the α- and β- caseins, as suggested by the work of McKenzie and Wake (1961). Alpha- and β-caseins precipitated out when the pH was decreased to 3.5. Following centrifugation the supernatant was made 41% with respect to alcohol and a kappa-rich precipitate formed (Figure 7-A).

When a slightly smaller starting volume was taken (900 ml) kappa-rich fractions were obtained at 41% ethanol and also at 50% ethanol concentrations (Figure 7-C and D). If the starting pH was lowered only to 8.5 instead of 3.5 (Figure 7-B), a purer kappa-fraction was obtained from the 41% alcoholic precipitate. The procedure is given in Figure 2.

There was some difficulty in reproducing the procedure, especially if the starting volume was changed. The final purification of this crude κ-casein by the method of McKenzie and Wake (1961) did not seem to eliminate the presence of the non-kappa-components.

From Preparative Horizontal Polyacrylamide Gel Electrophoresis

A method for obtaining smaller yields of relatively purer κ-casein by preparative horizontal PAGE appeared to be feasible. Samples in concentrations from 3-5% were PAUG electrophoresed, after which edge strips were removed and stained for protein bands. A stained strip is shown in Figure 8 and about four bands (as numbered) can be seen in the kappa-region. These bands were excised as a group and also as individual bands. The isolation procedure is given in Figure 3. After the PAG transverse section was excised it was homogenized in distilled water to disrupt the gel and increase the surface area. Allowing the gel slurry to stir overnight enabled the protein to diffuse into the distilled water. Centrifugation and filtration removed the large gel particles.

The isolated bands of protein were resolved on a SUG at pH 8.6 and the results are shown in Figure 9-A-F. In Slot A were proteins identified as bands 2, 3 and 4 in Figure 8 in their order from the origin with respect to the kappa-region. Slot B contained protein bands 1, 2, 3 and 4 of Figure 8 again in their order from the origin.

In the crude kappa-preparation represented by Figure 8 the first two bands from the origin are not thought to represent κ-caseins since they do not appear to be sensitive to rennin. Certain observations suggest that these two bands probably represent the gamma and temperature-sensitive caseins (McCabe and Brunner, 1966).

Slots C, D and E of Figure 9 show kappa-protein bands 2, 3 and 4 as marked in Figure 8. Slot F contained an acid extracted κ-casein preparation run to locate the band positions with respect to α- and β- caseins.

The proteins from bands numbered 2 and 3 of Figure 8 were obtained in very small amounts and showed a clouding effect when exposed to the action of rennin.

Since band 2 appeared to be more homogeneous it was eluted and run in the ultracentrifuge for sedimentation-velocity ($S_{20,w}$) and molecular weight determinations. The $S_{20,w}$ value for 4 mg/ml sample in 5 M guanidine at pH 6.4 (T = 0°) was 2.1 S while the value obtained at 5 mg/ml in a 5 M guanidine + 0.01% 2-ME, pH 6.1 (T = 0°) was 1.7 S. The molecular weight calculated for a 10 mg/ml sample in 5 M guanidine + 0.1% 2-ME (v/v), pH 8.1 (T = 0°) was 19,022. This value of 19,022 agrees with those reported by Swaisgood and Brunner (1963) of 18-20,000 for κ-casein in strong dissociating agents with 2-ME and with the value of 19,000 reported by Woychik *et al*. (1966) for κ-casein A-1 and κ-casein B-1.

Acid Extraction of Isoelectric Casein

While studying the acid extraction procedure of whole casein employed by Groves (1960), κ-casein was detected in the resulting supernatant.

This method appeared to offer a way of obtaining reasonably pure κ-casein without going through a series of rigorous chemical and physical manipulations. The underlying hypothesis was that some of the extreme measures as employed in most preparations may in fact alter the protein chemically or even denature it beyond recovery.

The basic scheme for the acid extraction of isoelectric casein is shown in Figure 4. One method of acid extraction involved only dilute acetic acid at a pH of 3.8-4.0. After the pH became rather constant at 3.8-4.0 the mixture was stirred overnight in the cold (0-5°). The low temperature should monomerize the β-casein and at the same time free kappa from the β-κ-casein complex.

The pH was readjusted, if necessary, to 3.8-4.0 and the solution was warmed to 35-40° and then filtered on Buchner funnel under reduced pressure while at this elevated temperature. Recent work by Kenkare and Hansen (1967); and Siegel and Lillevik (1967) indicate that high temperatures may enhance the dissociation of the α_s-κ-casein complex.

The resulting filtrate was clear. Prior decanting of the supernatant or centrifugation before filtration

possibly speeded the filtration process, but did not produce a clear filtrate. In this case the filtrate was milky white in color and contained large amounts of α- and β- caseins as determined by SUGE. To obtain a clear filtrate, a uniform mixture had to be poured into the Buchner funnel. Whatman No. 1 filter paper appeared to give a clear filtrate and a good rate of filtration. Whatman No. 42 filter paper seemed to become clogged and this caused the filtration rate to decrease substantially.

The clear filtrate (pH 3.8-4.0) was adjusted to a pH of 4.65 and allowed to stand overnight in the cold (0-5°) during which time a precipitate settled out. The resulting precipitate was removed by centrifugation in the cold (0-5°) and analyzed by SUGE (Figure 10-A). The kappa-region appeared as a smear even in the presence of Cleland's reagent. The crude k-casein obtained by acid extraction of isoelectric whole casein and precipitated at pH 4.54 was purified twice according to the method of McKenzie and Wake (1961). The SUG patterns of the product are shown in Figure 10-B, C. The PAUG contained Cleland's reagent on this product and thus its pattern shows the bands more distinctly then the SUG which contain no disulfide cleaving agent. As can be seen in the PAUG pattern the purified k-casein contained essentially three bands. Figure 11 shows an electrophoretogram of another acid extraction k-casein preparation before and after exposure

to the action of rennin. The smeared kappa-region disappeared and only one major p-k-casein band is present.

The sedimentation behavior of a 0.9% solution of this purified k-casein at pH 7.1 and 5° in a phosphate buffer ($\Gamma/2 = 0.1$) showed no material sedimenting more slowly than the fast sedimenting k-casein with a $S_{20,w}$ value of 13.9. This agrees with the values of 13.9 found by McKenzie and Wake (1961) and Swaisgood (1963).

The investigations of Purkayastha and Rose (1965) and Woychik et al. (1966) indicate that the major kappa-band contains no hexose or sialic acid. The pH 4.65 acid-extracted k-casein was PAG electrophoresed and stained for both protein and carbohydrates. The results are seen in Figure 12 and show that the carbohydrate moiety was uniformly distributed throughout the kappa-region. Since the GMP appears to be the major soluble rennin-reaction product it may be possible that carbohydrate components are being lost during the preparation and purification of k-casein.

Acid Extraction in the Presence of Sodium Chloride

When sodium chloride is included (10 g/l) in the acid extraction procedure for k-casein the resulting clear pH 3.8-4.0 filtrate shows no tendency to precipitate when adjusted to pH 4.65. However, when this solution was dialyzed at 0-2° against distilled water a precipitate occurred which was removed by centrifugation at 0-5°.

The SUG pattern of this precipitate is shown in Figure 13-A. The pattern shows a kappa-rich protein together with α- and β-caseins as contaminants.

For further purification the precipitate was redissolved in 1 M NaCl at a pH of approximately 6.0 and dialyzed exhaustively against distilled water in the cold (0-5°). The lower temperature was employed in an effort to reduce the β-casein contamination since β-casein monomerizes (and solubilizes) at low temperatures and would be therefore less apt to precipitate out. The precipitate which occurred upon this treatment was examined by SUGE and as Figure 13-B shows it appears like p-k-casein.

The sedimentation analysis pattern of a 1% solution of the material represented in Figure 13-B in 0.1 M veronal buffer, pH 8.6 ($\Gamma/2 = 0.1$) was similar to Figure 22-E and showed essentially no fast moving material. The $S_{20,w}$ was determined as 0.94 S which agrees with values of 0.9 S found in later experiments (Figure 30) with purified p-k-casein. As the preparation was treated with antibiotics, the formation of this p-k-casein-like material through bacterial action tends to be ruled out. Proteolysis by proteases contained in milk is possible, but so complete a transformation from kappa to p-k-casein was not observed at any other time during this investigation.

However, it seems that whenever sodium chloride is present in casein preparations the appearance of cathode

migrating components is to be expected. Somewhat similar results were found by Beeby and Nitschmann (1963) who observed the appearance of p-k-casein material through the use of concentrated urea solutions in the absence of rennin.

Preparation and Properties of the Glycomacropeptide

The preparation of the GMP is adapted essentially from the procedure of Alais (1956). In this study the pH of the three times reprecipitated isoelectric whole casein was maintained below 7.0 to prevent alkaline hydrolysis and possible loss of carbohydrate material. The major portion of the accompanying TCA was removed by extraction with organic solvents. The preparation scheme for GMP is shown in Figure 5.

Gel Electrophoresis

Discontinuous buffer system.--PAUGE (discontinuous buffer system) of the GMP showed only a single band moving with the migrating front. Periodate-fuchsin staining (PAS) procedures indicated that the carbohydrate residues were located in the same band.

Figure 14-A, B shows a crude and purified sample of the GMP, stained by amido black, where the protein is at a position on the migrating front.

Figure 14-C, F are PAGE patterns where the GMP sample was run in duplicate. The completed gel was cut

into two parts, one was stained for protein and the other for carbohydrates. The GMP band of Slot C of Figure 14 which was stained for protein is very difficult to see but is on the migrating front. Slot F is the same sample stained for carbohydrates and its band also lies along the migrating front. The gel shrinkage due to long submersion in alcoholic solutions is evident. Slots I-K are the same as Slots F-H Figure 14 which have now been counter-stained with amido black after PAS development to bring out the protein bands. Slot I shows the presence of protein material along the migrating front position.

The miscellaneous casein fractions in Slots G, H of Figure 14 and Slots J, K of Figure 14 are presented to point out the difference between the carbohydrate stain and the counter-stain with amido black and to also demonstrate that the original bands of Slots D, E (Figure 14) can be reproduced.

Continuous buffer system.--The feature of using a continuous buffer system is that it tends to greatly reduce the mobility of the protein through the gel.

When a PAG with continuous buffer (no urea) at pH 8.6 for electrophoresis is used, the GMP behaves quite differently. Two non-carbohydrate containing bands appear, one just ahead and one just behind the migrating front (which is BPB dye). The carbohydrate containing protein species is found about midway between the origin and the migrating front.

Figure 15-A displays the location of the two non-carbohydrate containing moieties; however, the resolution is not quite as sharp as with discontinuous buffer system. About midway between the origin and the front in Figure 15-A is an indefinite light smear, which in later patterns is ascertained to be the position of the carbohydrate containing species.

Figure 15-B represents the location of the carbohydrate containing protein species resolved in a continuous buffer PAG. Two bands are evident about midway between the origin and the final position of the migrating front. These two bands may well represent the genetic variation ascribed to k-casein.

Purification of the Glycomacropeptide

The GMP product was passed over a Bio-Gel P-2 column to remove salts and to attempt a separation of the GMP from other polypeptide fractions. The column had previously been calibrated for void volume and effluent volumes that would give salt free fractions of GMP. This calibration step proved unnecessary since the absorbance by GMP at 280 mμ is sufficient enough to monitor fractions produced. The elution pattern is shown in Figure 17. The two small peaks following the major peak in this figure are thought to be polypeptide fractions.

When the major protein peak of Figure 17 was re-analyzed by continuous buffer PAGE only the carbohydrate

moiety was observed. These patterns are shown in Figure 16. Slot A shows the purified GMP stained for protein and no protein bands appeared. This indicates the absence of the fast moving non-carbohydrate moieties. Slot B shows the location of the carbohydrate containing material. Slot C represents Slot B previously stained for carbohydrates and now counter-stained with amido black. The only protein material in evidence is in that region where carbohydrate material has also been shown to be present.

The purified GMP appeared to be free of non-carbohydrate containing material.

Physical Studies on the Glycomacropeptide

Sedimentation-Velocity Coefficients

The results of these experiments showed that the GMP sedimented as a single symmetrical peak. There was no appearance of any material sedimenting faster than the GMP. Figure 22-A shows the sedimentation pattern of a 6.1 mg/ml GMP sample in 0.1 M NaCl at pH 6.3, 20° run for 80 minutes. The sedimentation coefficients were determined for several concentrations of protein, corrected to water at 20° and extrapolated to infinite dilution. This data is reported in Table 2. Figure 19 demonstrates that at infinite dilution the GMP has a sedimentation coefficient of 1.2 S. This agrees with a value of 1.5 S as previously determined by Brunner and Thompson (1959).

Molecular Weight Determinations

Sedimentation-equilibrium experiments employing the short column techniques of Van Holde and Baldwin (1958) were used to determine the molecular weight of the GMP. These analyses were run at several concentrations and the apparent molecular weights obtained were then extrapolated to infinite dilution to obtain the true molecular weight. Table 2 shows data from these experiments and Figure 20 represents the plot of the apparent molecular weight versus concentration. The molecular weight at infinite dilution was found to be 7,500. Figure 22-B shows a sedimentation-equilibrium pattern for the 6.1 mg/ml sample in 0.1 M NaCl pH 6.3 at 20° and a rotor speed of 24,630 RPM. Molecular weights of 8,000 have been reported by Nitschmann and Henzi (1959), Wake (1959a) and Jollés et al. (1961) and 6,000 by Swaisgood (1963).

Diffusion Coefficient

Sedimentation-equilibrium experiments indicate a rather high diffusion rate for the short period of time (5-6 hours) required to reach apparent equilibrium. The diffusion constants were calculated according to the equation (Schachman, 1957):

$$\sigma^2 = (A/H)^2 \frac{1}{2t}$$

σ^2 is the square of the second moment and is plotted against time. The apparent diffusion constant for each

concentration is determined from the slope of this plot.

Extrapolation of apparent diffusion constants to infinite dilution give the true diffusion constant. The extrapolated diffusion constant was corrected for the viscosity of water and found to be:

$$D^{\circ}_{20,w} = 27.0 \text{ Fick Units}$$

The diffusion constant data is contained in Table 2 and the extrapolation plot is shown in Figure 21.

This value of 27 F. U. is quite high and does not agree with previously determined values of 7.5 Ficks by Brunner and Thompson (1959) and 10.0 Ficks by Swaisgood (1963).

If the sedimentation and diffusion data are combined into the Svedberg equation (Svedberg and Pedersen, 1940):

$$M = \frac{R\ T\ S}{(1 - \bar{v}p)D}$$

where M = molecular weight

R = gas constant, 8.314×10^{7} erg/mole/deg

T = absolute temperature

$\bar{v}$ = partial specific volume

p = density of the solution

D = diffusion constant

A molecular weight of 3,700 is obtained. This molecular weight value is approximately one-half that of 7,500 obtained by sedimentation-equilibrium experiments. However, the value of 3,700 is quite close to the minimum molecular weight of 4,000 estimated for the GMP by Kalan and Woychik (1965). Brunner and Thompson (1959) using a sedimentation and diffusion date in the Svedberg equation calculated a molecular weight of 15,260.

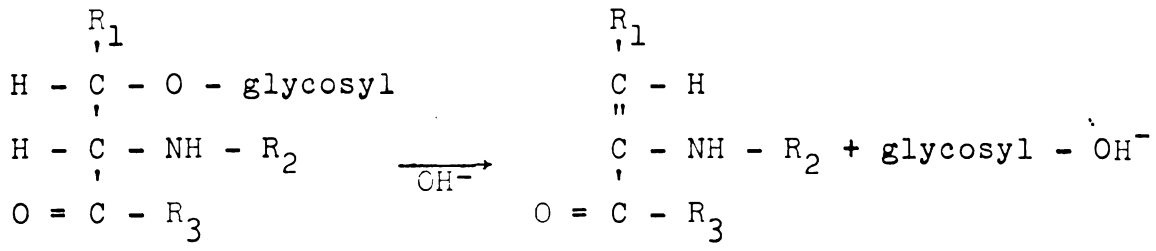
Free-Boundary Electrophoresis

The mobility of the crude GMP in veronal buffer ($\Gamma/2$ = 0.1) pH 8.6 was -5.51 Tiselius Units for the ascending pattern and -4.95 T. U. for the descending pattern. These values differ slightly from those of Brunner and Thompson (1959) who reported values of -6.18 and -4.62, respectively.

Chemical Studies on the Glycomacropeptide

The Beta-Elimination Reaction

The effect of sodium hydroxide on glycoproteins has been studied and reported by Carubelli *et al*. (1965) and discussed extensively by Neuberger, Gottschalk and Marshall (1966). If there are carbohydrate moieties linked by O-glycosyl bonds to threonine and serine then the following base catalyzed reaction may occur.



Since the dehydro amino acids show absorbance at 241 mμ wave length the course of the reaction can be easily followed.

Such a β-elimination reaction with GMP in 0.5 N NaOH was followed at 241 mμ for six hours (Figure 23) and terminated after 24 hours. The reaction mixture was neutralized, lyopholyzed, redissolved in water and passed over a Bio-Gel P-2 column to remove salts and cleaved carbohydrates. The first fraction following the void volume was collected and it can be seen from Figure 18 that there were no significant second fraction.

Estimated from the absorbance vs time plot (Figure 23), the reaction appeared to have reached maximum after six hours and was allowed to continue for 24 hours to insure completeness. The base treated GMP was found to have lost 52% of its total hexose content.

Beta-elimination will not occur if the O-glycosyl bond is attached to N - or C - terminal amino acid residues of a peptide because of inductive and resonance effects. Since approximately 50% of the total hexose was found after 24 hours exposure to alkaline condition it may be concluded

that the remaining hexoses are terminally attached. This would lend support to the work of Jollés et al. (1962) wherein they indicated a possible bond between phenylalanine of p-k-casein and a carbohydrate group on the GMP.

Chemical Composition of Glycomacropeptide

The results of the compositional analysis are shown in Table 3 and show a wide difference in many of the specific analyses.

The phosphorous content of 0.72% is approximately twice that reported by Jollés and Alais (1960).

The total hexose content of 4.24% is comparable to the 5% found by Brunner and Thompson (1959), but less than the 7.4% found by Jollés and Alais (1960).

The hexosamine content of about 4% occurrs about midway between the 2.3% found by Brunner and Thompson (1959) and the 6.5% found by Jollés and Alais (1960).

The sialic acid content of 8.15% is considerably lower than the values of 11.5% found by Brunner and Thompson (1959) and the 14.3% found by Jollés and Alais (1960).

A tryptophan content of 0.05% was considered insignificant.

Ultra Violet Spectrum

The purified GMP product (0.3 mg/ml in 0.1 N NaCl) showed an absorbance of 0.44 at 280 mμ (Figure 24). There is also a strong absorbance at 260 mμ.

Amino Acid Analysis

The results of the amino acid analysis upon GMP are shown in Table 4. Comparison of this study's amino acid analysis with those of Jollés et al. (1962), Kalan and Woychik (1965) and DeKoning et al. (1966) shows much agreement.

If all the individual amino acid residues are multiplied by their respective residual molecular weight (molecular weight of amino acid minus 18) the peptide total molecular weight is 5932.

No determination was performed for ammonia, but DeKoning et al. (1966) found thirteen moles of ammonia which if added to the previous value will give a total molecular weight of 6053.

This molecular weight of 6053 constitutes only the peptidic portion of the molecule. Based on chemical analysis, at least 17.24% is non-peptide, leaving 82.76% of the GMP as polypeptide.

When the non-peptide fraction is added to the peptidic portion one obtains a molecular weight of 7,435 grams for the complete GMP. This value of 7,435 taken into account the amino acids, ammonia, phosphorous and carbohydrate residues.

Interpretation of the Glycomacropeptide Study

The results of the study upon the GMP product of rennin action with whole casein indicate that the 12% TCA-soluble fraction contains non-carbohydrate peptides in addition to the GMP. Resolution of the 12% TCA-soluble fraction on Bio-Gel P-2 removes the non-carbohydrate components and indicates that their molecular weights were less than 3,600. With a molecular weight of less than 3,600, the possibility that this non-carbohydrate material represents the GMP with its carbohydrates removed seems unlikely.

There was a high degree of correlation between the molecular weight obtained by ultracentrifuge studies, 7,500, and that obtained by amino acid plus prosthetic group content analysis, 7,435. The molecular weight of the GMP based on the results of this study is 7,500.

The ultra violet spectrum indicated absorbance not only at 280 mμ but also at 260 mμ. As no aromatic amino acids have been reported for the GMP, the absorbance at 280 mμ is probably due to carboxyl groups within the molecule itself. The strong absorbance at 260 mμ could be due to the presence of the carbohydrate material, especially the sialic acid residues.

Approximately 50% of the total hexose present was removed by alkaline hydrolysis. Spectrophotometric observations indicate that a β-elimination type reaction

mechanism was responsible for some or all of the hexose lost. The remaining hexose is thought to be located in an N - or C - terminal position of the remaining polypeptide which would make it resistant to nucleophilic attack.

Chemical composition analysis based on a mole as equivalent to 7,500 grams reveal that the GMP contains two moles each of phosphorous, hexose, hexosamine and sialic acid.

Thus the GMP is visualized as a molecule whose molecular weight is about 7,500 and contains at least two carbohydrate chains. These chains are probably linked to the peptide portion through the hydroxyl groups of serine or threonine. Each carbohydrate chain probably consists of phosphorous, hexose, hexosamine and sialic acid. One-half of the carbohydrate chain could be located in a terminal position and may be involved in the linkage to p-k-casein in the original k-casein molecule.

Preparation and Properties of Para-Kappa-Casein

From Isoelectric Casein

The SUG electrophoretograms of p-k-casein produced by rennin action on isoelectric casein indicate the presence of α- and β-casein components along with several undefined protein bands. Figure 25-A, B shows the considerable amount of non-p-k-casein components actually present in its preparation from isoelectric whole casein.

From Calcium-Treated Casein Solutions

Isoelectric casein solutions at pH 6.5 was made 02.5 M with respect to calcium, while keeping the pH constant at 6.5. Two fractions were obtained after centrifugation, namely the calcium insoluble fraction and the supernatant.

The supernatant was treated with rennin and the resulting p-k-casein analysis is shown in Figure 26. These SUG electrophoretograms show that the resulting p-k-casein still contained a large amount of α- and β-fractions.

The 0.25 M calcium insoluble fraction was suspended in water, stirred for 15 minutes and then centrifuged and filtered. The filtrate contained α-, β- and k-caseins as shown in Figure 33-A. This water soluble extract exhibited a positive rennin reaction and produced a crude p-k-casein product.

Similar results were obtained from the precipitate remaining after extraction at pH 3.8-4.0. The precipate was dissolved at pH 6.5 and treated with calcium and rennin as just described. The resulting p-k-casein still contained α- and β-caseins as contaminants.

From Acid Extracted Kappa-Casein Preparation

P-k-casein resulting from the action of rennin on crude kappa prepared by the acid extraction of isoelectric casein earlier described was also found to be impure.

This result is shown in Figure 11-B. For use as a starting material in the preparation of crude p-k-casein, it appeared to yield the smallest amount of non-p-k-casein contamination and the least number of contaminating proteins.

The gamma and temperature-sensitive caseins (McCabe and Brunner, 1966) found just on the anode side of the origin are not present to any great degree as seen in Figure 11-B. These proteins were found to be insensitive to rennin and also proved to be quite troublesome to remove in subsequent purification steps.

Purification of Para-Kappa-Casein

Solubility Differences

The purification of p-k-casein by utilizing its solubility differences indicated that some of the contaminants were solubilized and could be removed. P-k-casein was also found to be slightly soluble but only to a very small extent. The efficiency of this method under the conditions employed as seen in Figure 25-C suggested that a purification effort would better be continued in other directions.

Column Chromatography

Crude p-k-casein produced from acid extracted k-casein and rennin was passed over a column of Bio-Gel P-20, which was equilibrated with a 5 M urea solution containing

0.1% of 2-ME. This gel has an exclusion molecular weight of 20,000 which should hold up the p-k-casein while allowing the other casein fractions to pass through. The results of this fractionation are shown in Figure 22. Slot A represents the starting material of crude p-k-casein, prepared from acid-extracted k-casein. Slots B-F represent peak fractions 1, 2, 3, 4 and 5 respectively during elution and were analyzed on SUG (+2-ME) at 1% protein concentrations.

The increasing density of the p-k-casein bands can be noted as the fraction peaks increase. There also appears to be an increase in the number of p-k-casein bands with fraction number as well as an increase in the density of the slower moving p-k-casein bands. Thus the two major p-k-casein bands which appear in the original material (Slot A) seem to migrate faster than those in the purified sample represented in Slot E.

Several factors made this method unreliable as a preparative procedure for p-k-casein. Since the urea-2-ME solution has a very high absorbance at 280 mμ alone, false peaks were obtained whenever freshly made eluting solutions were added during the course of chromatographic purification experiments. Complete elution of the column required washing with large volumes of solvent and even then enriched p-k-casein fractions were obtained in the early cuts of succeeding runs. The yields of product

represented by Figure 27 Slot E were small and the highly purified p-k-casein fraction contained other casein contaminants.

Polyacrylamide Disc Gel Electrophoresis

The p-k-casein preparation was dissolved in a Tris 5 M in guanidine stock solution containing 0.1 mg/ml of Cleland's reagent. A drop or two of the amido black dye solution was added to provide a method of following the migrating front. Amino black dye solution was earlier found to migrate with the front in horizontal PAG and SUG employing discontinuous buffer systems.

At a pH of 8.6 electrophoresis on PAG and SUG, the p-k-casein migrates toward the cathode. PAG disc electrophoresis effected a purification of crude p-k-casein by allowing all the anode migrating species to move into the gel and leaving behind the p-k-casein. The results of this type of purification are shown in Figure 11. Slot A is the crude kappa-fraction and Slot B is the crude p-k-casein fraction resulting from rennin on the crude kappa-fraction of Slot A. Slot C is the disc preparative PAG electrophoretically purified p-k-casein.

P-k-casein is an ideal protein to purify by the polyacrylamide disc gel technique because of its cathodic migration at pH 8.6 in gels. Its insolubility in non-dissociating systems prevents movement into the

cathodic buffer layer and possible precipitation on the cathode electrode. A relatively large sample (200-300 mg) could be applied to the preparative gel for purification.

The actual number of initial or nascent p-k-casein bands appears to be one (Figure 11-B). When the k-casein-rich fraction is prepared by the acid-extraction procedure, only one p-k-casein band appears. When this p-k-casein undergoes any more purification, two protein bands were observed (Figures 11-C and 28-A, B). The appearance of these bands upon purification may be explained as the result of random polymerization or aggregation of the nascent p-k-casein. Their migration in SUG would be slowed down due to the sieving action of the starch network. The number of protein bands migrating toward the cathode has been observed to be as high as nine although usually there are only one to three major bands (Figure 29).

Physical Studies of Para-Kappa-Casein

If the protein solutions of p-k-casein were allowed to dialyze against the solvent even for short periods of time, a distorted initial concentration pattern in the ultracentrifuge was observed (Figure 22-C) as compared with the normal pattern (Figure 22-D). Normal initial patterns were obtained when the protein was sedimented within a short time after dissolving and without dialysis. All p-k-casein ultracentrifuge studies had to be done in this

fashion. No correction was made for charge effects or specific protein solvent interaction.

The results of the physical studies are shown in Table 5.

Sedimentation-Velocity

The sedimentation-velocity experiments on p-k-casein were performed at 20° in 5 M guanidine pH 8.1 containing Cleland's reagent (0.1 mg/ml). The sedimentation behavior indicated a symmetrical peak (Figure 22-E) with no material moving faster than the p-k-casein. The sedimentation coefficients were determined for a series of concentrations, corrected to water and extrapolated to infinite dilution (Figure 30). The $S^{\circ}_{20,w}$ was found to 1.25 S.

Molecular Weights

The apparent molecular weight of various reduced p-k-casein concentrations was determined by employing the short column method of Van Holde and Baldwin (1958). The results were extrapolated to infinite dilution to give a molecular weight of 14,000 (Figure 31). Some polydispersion is evident in the equilibrium sedimentation pattern (Figure 22-F) and is substantiated by the Mz/Mw ratio 1:3. The conclusion may be that all the disulfide bonds have not been reduced and thus there are some molecular species present with molecular weights greater than the 14,000 obtained by equilibrium experiments.

Approach-to-equilibrium experiments employing Trautman plots as modified by Erlander and Foster (1959) showed that the lightest component present had a molecular weight of 13,600.

Diffusion Constants

Apparent diffusion constants of 11.3 and 11.5 Fick units were calculated for p-k-casein solutions containing 5.1 and 6.1 mg/ml of protein, respectively. These values when substituted into the Svedberg equation yield molecular weights of 14,600 and 14,200.

Chemical Studies of Para-Kappa-Casein

Compositional Analysis of Para-Kappa-Casein

The results of the compositional studies are shown in Table 6. P-k-casein was found to contain 0.02% phosphorous, 2.3% hexose, 0.06% hexosamine, a trace of sialic acid and 0.7% tryptophan. This accounts for about 3% of the p-k-casein monomer molecule.

Amino Acid Analysis

The amino acid analysis results are given in Table 7. When the number of residues found for a specific amino acid was multiplied by their respective residue molecular weight (molecular weight of the amino acid -18) and then summed, a molecular weight of 10,856 is obtained.

Since no specific analysis was carried out for cystine and ammonia the results of other investigators were added into this study. The residue molecular weight of two moles of cysteine, twelve moles of ammonia and since the tryptophan analyses were low when compared with other reports, two moles of tryptophan were included. This gives a molecular weight of 11,634 for the peptidic portion of the p-k-casein molecule. The peptidic portion of p-k-casein represents 97% of the molecular weight. The total monomeric molecular weight of p-k-casein, including the 3% of carbohydrates and phosphorous is 11,994.

The result of the amino acid analyses differs considerably from those of other investigators. The estimation of residue concentrations was based on the recovery of the internal standard norleucine and all calculated values were rounded off to the nearest integer.

Jollés et al. (1963) and DeKoning et al. (1966) based their residue calculations on an assumed molecular weight of p-k-casein of 20,000. When their results are totaled molecular weights of the peptidic portion of p-k-casein are 20,130 and 19,436 respectively. Agreement between the amino acid analysis of this study and those of Jollés et al. (1963) and DeKoning et al. (1966) can be found when their results were corrected to a molecular weight of 11,634 for the peptidic portion of p-k-casein. These results are given in Table 8.

Kalan and Woychik (1965) based their amino acid residue calculations on the ratios of eight amino acids. The sum of their amino acid residues indicates a molecular weight of 17,360 for the peptidic portion of p-k-casein and does not include tryptophan or ammonia residues.

Interpretation of the Para-Kappa-Casein Study

The results of the study with p-k-casein indicated that this component can be prepared from a variety of casein sources, including calcium-sensitive casein thought to be devoid of k-casein.

The purified p-k-casein had a $S^{\circ}_{20,w}$ of 1.25 S. The molecular weight by sedimentation-equilibrium methods was found to be 14,000 with the lightest component about 13,600 as determined by modified Trautman plot methods. The molecular weight obtained by the combination of sedimentation and diffusion data was 14,600 and 14,200. The molecular weight as determined from amino acid analysis was 12,000. The monomeric molecular weight of p-k-casein based on this study is presumed to be 13,600 $\pm$ 1,000.

Studies by Swaisgood and Brunner (1963) produced a molecular weight of 18-20,000 for reduced k-casein. Woychik et al. (1966) found that the major k-casein components when reduced had a molecular weight of 19,000. The purified k-casein studied in this investigation indicated a molecular weight of 19,022. Thus it would appear that the molecular

weights of 17-20,000 for p-k-casein as indicated by the amino acid analysis of other investigators would be slightly high. None of the previous investigators have demonstrated any degree of purity of their preparations. P-k-casein prepared by a variety of methods as given in this study indicate that these methods required considerable purification.

The compositional analyses indicate the presence of one mole of phosphorous and one mole of hexose based on the tentative molecular weight of 13,000 $\pm$ 1000.

The nascent state of p-k-casein manifests itself as one fast cathodic migrating band on SUGE. Based on the work of Hill (1964) and Beeby (1964) who found only cysteine in casein micelles and k-casein, it could be hypothesized that this nascent p-k-casein contained only cysteine. The continued chemical manipulation appears to bring about oxidation of thiol groups which results in intermolecular disulfide bonding as well as disulfide interchange. This alteration is indicated on SUG by the progressive development of two or more rather slow cathodic moving bands, although as many as nine zones have been observed migrating in the cathode direction (Figure 29).

Support for this hypothesis can be observed in the chromatographic purification of p-k-casein (Figure 27). As the more enriched p-k-casein was eluted from the column, the number of slower moving cathodic bands increased. The highly enriched p-k-casein fraction (Slot E) was exposed to

urea-2-ME for the longest period of time allowing for complete reduction of any disulfide. Thus upon dialysis a more random type of intermolecular disulfide bonding could be expected. In more dilute p-k-casein solution this intermolecular disulfide may be achieved in a more ordered fashion. The random type intermolecules could lend itself to the aggregation of large molecules which would then migrate more slowly due to the sieving action of the starch gel.

Study of the Enzyme Rennin

Starch-Urea Gel Electrophoresis

The SUG electrophoretogram of rennin is shown in Figure 32. Three or four light bands having a faster migration that precedes the main rennin band. One or two very faint bands may be seen migrating at a slower rate. These faster and slower bands may be contaminants or isoenzymes. No effort was made to determine either possibility. Based on visual observation of the gel pattern these miscellaneous bands appear to constitute less than 10% of the major species regardless of their identity.

Ultracentrifuge Studies

The sedimentation behavior of a 1% rennin solution in 5 M guanidine at pH 6.0 and 0° C. showed a symmetrical peak with essentially no material moving faster than rennin. The sedimentation coefficient when corrected to water at 20° was 3.2 S.

The molecular weight of a 1% solution in 5 M guanidine pH 6.0 and 0° C. was determined using the short column technique of Van Holde and Baldwin (1958), and a value of 31,920 was obtained.

This agrees with values of 34,000 of found by by Djurtoft, Foltmann and Johansen (1964) using sedimentation and diffusion data and 31,000 found by Andrews (1964).

The enzyme solution appeared to be monodispersed and the z-average molecular weight (Mz) was 33,257, which gives a Mz/Mw ratio of about one.

Reaction of Rennin with Miscellaneous Alpha- and Beta-Rich Fractions

Alpha- and β-rich casein fractions were treated with rennin, then the reaction samples were frozen and lyopholyzed. The SUG electrophoretograms of the products are shown in Figure 34.

All samples exposed to rennin show several identical features. The β-casein appears to migrate at a slightly faster rate and the α region seems to split into several components after treatment with rennin. The rennin treated samples were run in 3% concentration, while those of the untreated were 2% and yet the β-casein region appears to be greatly diminished in the rennin treated samples.

Reaction of Rennin with Calcium-Precipitated Casein

The calcium precipitated casein from the acid extracted k-casein preparation was subjected to rennin reaction. DFP was added to the casein solution prior to the addition of rennin to inhibit any trypsin which may have been present in the rennin preparation. After ten minutes the reaction mixture was frozen and lyopholyzed.

The PAG electrophoretogram is shown in Figure 33. Slot (Figure 33-A) shows the starting material to contain α-, β- and some k-casein. Slot B (Figure 33) shows the α-casein area has split into three components and that the β-region had migrated at a faster rate and is also greatly diminished in area. Both samples are 1% protein solutions. The results are quite similar to those found in the previous solutions. The results are quite similar to those found in the previous experiment with the miscellaneous α- and β-rich fractions (Figure 34). While not clearly visible there are two faintly stained bands moving toward the cathode.

The PAG was run in duplicate and cut into two halves. One-half was stained for protein and is represented in Figure 33. The other half was placed in distilled water for two hours to remove excess urea and then immersed in a 0.25 M calcium chloride solution as described by McCabe and Brunner (1966).

The α- and β-regions of Slot 33-A showed precipitation bands corresponding to their areas. No precipitation was observed in the k-casein region.

Figure 33 Slot B showed all three bands preceding the β-casein band precipitated as well as the β-casein band itself. The concentration of the protein solutions was 1% which made the precipitated protein band difficult to photograph and thus they are not shown.

Rennin appears to change the SUG electrophoretic behavior or undissociated α-, α_s- and β-caseins. The undissociated α- and β- appear to be slit into several components. Rennin acts on β-casein to produce a reduction in area and a slight increase in mobility. There is the possibility that β-casein may also be split into two fractions and thus the loss of area could be rationalized. All components split from the α- and β-caseins were found to precipitate in PAG when such gels were immersed in a 0.25 M calcium solution.

This action of rennin on undissociated α-, α_s-, and β-caseins probably indicates that some k-casein may be present and tightly bind these components in an α_s-κ- and β-κ-complex still precipitated by Ca^{++}. The results tend to lend some credence to the work of Lahav and Bahad (1964) and their conclusions that there may be several different calcium-insensitive components which are rennin-sensitive and associated with α- and β-caseins. But there is also

the possibility that α- and β-caseins may also be subject to cleavage by rennin.

SUMMARY

Purified k-casein in strong dissociating and disulfide cleaving agents was found to have a molecular weight of 19,000 and a sedimentation coefficient of 1.7 S. The addition of sodium chloride to k-casein solutions caused the precipitation of a p-k-casein-like material. This material had a sedimentation coefficient of 0.9 S.

The 12% TCA-soluble fraction was found to contain at least two non-carbohydrate containing components in addition ot the GMP as determined by PAGE. The 12% TCA-soluble fraction was passed through a Bio-Gel P-2 column which separated the non-carbohydrate material from the GMP. The GMP was run on PAGE and stained for protein and also for carbohydrate material. Ultracentrifuge studies indicate the GMP has a molecular weight of 7,500 and a sedimentation coefficient of 1.2 S. Amino acid analysis revealed that the GMP contained no aromatic amino acids. The molecular weight based on the amino acid analysis was 7,400. Compositional analysis indicated that the GMP contained two moles each of the following; phosphorous, hexose, hexosamine and sialic acid. One-half of the hexose content can be removed by alkaline hydrolysis through a β-elimination type mechanism. The GMP is postulated to contain

at least two carbohydrate chains. One chain appears to be in a terminal position since it is resistant to nucleophilic attack. This terminal carbohydrate chain may be involved in a linkage between the GMP and p-k-casein. The ultra violet spectrum of the GMP showed absorption at 280 mμ and a strong absorption at 260 mμ.

The results of this study indicate that p-k-casein can be prepared from a variety of casein sources, including the calcium-sensitive casein thought to be devoid of k-casein. A method for purification of p-k-casein by disc PAGE was developed. Ultracentrifuge analysis indicates that p-k-casein in 5 M guanidine and 0.1% 2-ME had a $S^{\circ}_{20,w}$ of 1.25 S and a molecular weight at infinite dilution of 14,000. Approach-to-equilibrium experiments revealed that the lightest component in the p-k-casein had a molecular weight of 13,600. The diffusion constants for 5.1 mg/ml and 6.1 mg/ml of p-k-casein were determined to be 11.3 and 11.6 Fick Units, which when coupled with sedimentation data yielded molecular weights of 14,600 and 14,200. Amino acid analysis of the p-k-casein indicated that the molecular weight of p-k-casein was 12,000. Compositional analyses indicate the presence of one mole of phosphorous and one mole of hexose based on a tentative molecular weight of 13,000 ± 1000. Nascent p-k-casein appeared as one fast cathodic migrating major band on SUG. After purification procedures two or more major bands were observed.

The enzyme rennin displayed a molecular weight of 31,900 and a sedimentation coefficient of 3.2 S. The SUG electrophoretograms of rennin indicate one major band and several lighter bands migrating somewhat faster. This study provided visual evidence of changes in α- and β-caseins as a result of rennin action.

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APPENDIX

GLOSSARY OF SYMBOLS AND TERMS

AB: Amido black dye used to stain protein zones.

Alpha-casein (α-casein): A casein component comprising 50% of the total casein. Alpha-casein can be separated into calcium-sensitive (α_s-casein) and calcium-insensitive (crude k-casein) fraction.

α_s-casein: The calcium-sensitive fraction of α-casein.

Beta-casein (β-casein): A component of casein comprising approximately 30% of the total casein.

BPB: Bromo phenol blue dye used as migrating front marker.

Cleland's reagent: dithiothreitol.

DFP: diisopropylfluorophosphate.

EDTA: ethylenedinitrolotetra acetic acid.

F (F. U.): Fick units; 1×10^{-7} cm^2/sec.

Glycomacropeptide (GMP): A primary reaction product from the action of rennin on k-casein and soluble in 12% (w/w) TCA.

Isoelectric casein: Whole casein which has been redissolved and reprecipitated at pH 4.6.

Kappa-casein (k-casein): The calcium-insensitive fraction of -casein and the primary substrate for the enzyme rennin.

2-ME: 2-mercaptoethanol.

PAG: polyacrylamide gel.

PAGE: polyacrylamide gel electrophoresis.

Para-kappa-casein (p-k-casein): A primary reaction product from the action of rennin on k-casein.

PAS: periodic acid--Schiff base development of gel glycoprotein zones.

PAUG: polyacrylamide gel with urea.

PAUGE: polyacrylamide gel urea electrophoresis.

Rennet: Sodium chloride extract from the fourth stomach of calves and represents a crude preparation of the enzyme rennin.

Rennin: a purified crystalline form of the enzyme rennin.

S (S. U.): Svedberg unit; 1 x 10^{-13} sec.

SGE: starch gel electrophoresis.

SUG: starch-urea gel.

SUGE: starch-urea gel electrophoresis.

TAME: N, N, N, N-tetramethylethylenediamine.

TCA: trichloroacetic acid.

Tris: tris(hydroxymethyl)aminomethane.

T (T. U.): Tiselius unit of electrophoretic mobility; 1 x 10^{-5} cm^2 sec^{-1} $volt^{-1}$.

Whole casein: The protein precipitated from skim milk at pH 4.6.

WRT: with respect to.