THE ISOLATION AND PHYSICAL-CHEMICAL CHARACTERIZATION OF A GLYCOPROTEIN FROM THE PROTEOSE-PEPTONE FRACTION OF COW'S MILK

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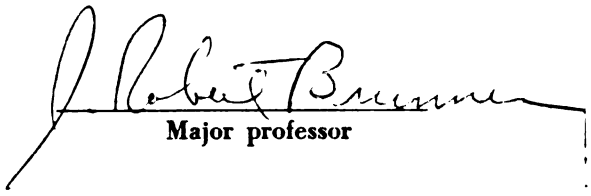

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ABSTRACT

THE ISOLATION AND PHYSICAL-CHEMICAL CHARACTERICATION OF A GLYCOPROTEIN FROM THE PROTEOSE-PEPTONE FRACTION OF COW'S MILK

by Wesu C. Ng

The proteose-peptone fraction of milk is a group of heat-stable, minor proteins which are not precipitated by heating skimmilk to 95° C for 30 minutes and subsequent acidification to pH 4.7. Three main components of the proteose-peptone fraction have been designated as components 3, 5 and 8 in free-boundary electrophoretic patterns. Component 3 referred to in this study is the slowest moving, electrophoretically homogeneous component in acrylamide gel electrophoretograms.

Procedures were developed for isolating component 3 from both heated and unheated skimmilk. An enriched preparation of component 3 was obtained from the acid-whey supernatant of heated skimmilk by fractionation with ammonium sulfate (55% saturation). The precipitate obtained contained component 3 in high concentration. Similarly, an enriched component 3-preparation was obtained from unheated skimmilk by fractionation of the crude globulin fraction with ammonium sulfate. Electrophoretically homogeneous preparations were isolated from the enriched preparations by a preparative-scale acrylamide gel electrophoretic technique.

A distribution study of the proteose-peptone components in whey and casein by ultracentrifugal methods and isoelectric precipitation indicated that component 3 was present only in whey and mainly in the classical lactoglobulin fraction.

Chemical analysis showed that component 3 was low in nitrogen (13.1%) and high in carbohydrate (17.2%). The carbohydrate portion contained 7.2% hexose, 1.0% fucose, 6.0% hexosamine and 3.0% sialic acid. The carbohydrate moities were identified by paper chromatography and consisted of galactose, mannose, glucosamine, galactosamine and sialic acid. The phosphorus and sulfur contents were 0.5 and 0.59 per cent respectively. Amino acid analyses revealed that component 3 was low in the aromatic amino acids tyrosine and phenylalanine; in the sulfur containing amino acids (no cysteine and cystine, low methionine); and high in glutamic acid, lysine and leucine. The chemical compositions of component 3, from heated and unheated skimmilk, were similar. The ionic mobility of component 3 in veronal buffer (pH 8.6, ionic strength = 0.2) was 3.5 Tiselius units in the descending pattern. The estimated isoelectric point from free-boundary electrophoretic experiments was pH 3.7. The sedimentation coefficient in veronal buffer (pH 8.6, ionic strength = 0.1) was estimated at 4.0 S units at infinite dilution. The diffusion coefficient in the same veronal buffer was estimated at $D^{o}_{20} = 1.8 \times 10^{-7}$ cm^2/sec at infinite dilution. Sedimentation-equilibrium studies showed that the weight-average molecular weight of component 3 in veronal buffer (pH 8.6, ion

strength = 0.1) was concentration dependent. The polymer-monomer equilibrium was shifted toward the light component in the presence of a dissociating system such as 5 M guanidine hydrochloride. The equilibrium molecular weight in veronal-5 M guanidine hydrochloride was estimated at 40,000 at infinite delution.

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By

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INTRODUCTION

The proteose-peptone fraction of milk is a group of heat stable, minor proteins remaining in the supernatant after skimmilk has been subjected to heat treatment (95° C for 30 minutes) and subsequent adjustment to pH 4.6. The protein fraction occurs in low concentration compared with the principle milk proteins such as casein or β-lactoglobulin.

Classically, proteose-peptone is referred to as a secondary or derived protein formed by hydrolysis of protein. Whether heating of skimmilk results in the hydrolysis of peptide bonds of the native milk proteins is not well established. The prevalent concept of the effect of heat on protein concerns the rearrangement of the tertiary structure of the protein molecule, rather than cleavage of the peptide bonds. Hence, the term proteose-peptone, as described in this study, is used to designate a particular milk protein fraction and is not intended to imply that it is a group of low molecular-weight, breakdown products of milk proteins resulting from heat treatment.

Previous studies indicated that proteose-peptone is a group of heterogeneous proteins. In order to best study the proteose-peptone components, a reliable analytical technique needs to be developed for detecting the individual components. Acrylamide gel electrophoresis and starch-urea gel electrophoresis were found to be quite satisfactory

for this purpose.

For my study, I chose to work with the slowest moving component of proteose-peptone in gel electrophoresis. This component may be analogous with the component 3 observed in free-boundary electrophoretic pattern of the proteose-peptone fraction. In later discussions, component 3 is referred to as the slow-moving component of the proteose-peptone group. Since it has not been established if component 3 exists in the native skimmilk, attempts were made to isolate component 3 from heated and unheated skimmilk.

In view of the above discussion, the present study seeks to work on the following objectives: 1) to prepare workable quantities of component 3, both from heated and unheated skimmilk; 2) to obtain chemical compositional data of both; and 3) to obtain physical parameters such as molecular weight, sedimentation coefficient, diffusion coefficient and others. For the purpose of organization, the thesis has been divided into two parts: Part I describes the preparation of component 3 while Part II evaluates the physical-chemical parameters of the preparations.

REVIEW OF LITERATURE

The term proteose-peptone was first designated by Rowland (1938) as the milk protein fraction which was not precipitated by heating skimmilk at 95° C for 30 minutes and subsequent acidification at pH 4.7, but precipitated by trichloroacetic acid at a concentration of 8%. Rowland referred to this group of proteins as the "secondary" proteins of a proteose-peptone nature which are present, together with the albumins and globulins, in the whey fraction of normal cow's milk. He reported a procedure for estimating the proteose-peptone content of milk based on nitrogen determination. The proteose-peptone nitrogen was calculated as the difference in the nitrogen contents between casein-free, non-coagulable heated-sera and that portion of the sera remaining in the supernatant at 8% trichloroacetic acid concentration, i.e., the non-protein substances. He reported that the soluble protein fraction or whey of normal milk was composed of approximately 76% albumin and globulin and 24% proteose-peptone substances.

Aschaffenburg (1946), studying the surface activity of milk proteins, obtained a protein fraction from acid or rennet whey by salting-out the sera obtained after the removal of casein and heat-coagulable proteins with ammonium sulfate at half saturation. He called this protein fraction sigma proteose because of its pronounced surface activity. A nitrogen analysis of sigma proteose showed that it had a

markedly reduced nitrogen content compared with other principle milk proteins. He first reported the heterogeneity of the fraction when it was shown that the fraction contained three electrophoretic peaks in phosphate buffer at pH 8.0 in the Tiselius cell. The three components, in decreasing order of mobility, and based on peak area, accounted for 10.5, 82.5 and 7.0%, respectively, of the total protein in sigma proteose. The ultracentrifugal data showed that this protein fraction was heterogeneous.

Weinstein, Duncan, and Trout, (1951) isolated a protein fraction from heated, rennet whey by a procedure similar to that of Aschaffenburg which they called the "minor-protein" fraction. They indicated from elementary analysis that the minor-protein fraction was different from sigma proteose. The nitrogen content of the minor-protein fraction, 10.0%, was low compared with the reported value of 13.95% N for sigma proteose. Their electrophoretic data showed that at least two components were present in the minor-protein fraction. They estimated the isoelectric zone of the major components of the minor-protein fraction at pH 3.7 to 4.4, based on electrophoretic mobilities at various pH values.

Larson and Rolleri (1955) made a systematic study of the effect of heat treatment on the electrophoretic mobilities of serum whey proteins. They noted that the unheated whey proteins on free-boundary electrophoresis showed a series of peaks, some barely visible due to the

predominance of other peaks. However, with increasing heat treatment, they observed a progressive decrease in the relative peak areas of the heat-denaturable whey proteins and an increase in the peak areas of the heat-stable whey proteins; until at 95° C for 30 minutes, only 3 components appeared in the electrophoretic pattern. By measuring the electrophoretic mobilities of the components appearing in the electrophoretic patterns of both heated and unheated whey, they accounted for a total of eight peaks, designated as component 1 through 8, in an increasing order of mobility. The three heat-stable components corresponded to component 3, 5 and 8 in the electrophoretic pattern, with component 5 constituting the major fraction. Components 3, 5 and 8, by nature of the preparation, are similar to the Rowland's proteose-peptone fraction. In the electrophoretic pattern of unheated whey, component 3 was obscured by the immune globulins (component 1 and 2); component 5 was partially obscured and appeared as a small peak or asymmetry on the side of β-lactoglobulin peak (component 6); component 8 was also partially obscured by β-lactoglobulin and serum albumin (component 7). The calculated electrophoretic mobilities of Component 3, 5 and 8 in veronal buffer (ion strength = 0.1; pH 8.6) were -3.0, -4.6 and -7.9 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, respectively. Since proteose-peptone components appeared in the electrophoretic pattern of unheated whey, Larson and Rolleri (1955) suggested that the proteose-peptone fraction was present in unheated skimmilk.

Jenness (1959) obtained a protein fraction rich in whey component 5 from unheated skimmilk. Casein and presumably the proteose-peptone fractions were salted-out of skimmilk saturated with sodium chloride. The enriched component 5 obtained with this procedure showed a major peak with an electrophoretic mobility of -4.5×10^{-5} cm^2 $volt^{-1}$ sec^{-1} at pH 8.6 in veronal buffer, which agreed well with the electrophoretic mobility of component 5 of the proteose-peptone fraction. It is interesting to note that in Jenness' procedure, the proteose-peptone components were obtained from fractions associated with micellar casein, while previously, the proteose-peptone components were shown to be present in the whey protein fraction.

Thompson and Brunner (1961) characterized several minor protein fractions of bovine milk, following previously known procedures for preparing the fractions. These included the proteose-peptone fraction of Rowland's, Aschaffenburg's sigma proteose, the minor protein fraction of Weinstein, Jenness' milk component 5 concentrate, and the soluble membrane-protein. They showed that these fractions were characteristically low in nitrogen (10-14%), high in ash (3-7%) and phosphorus (0.6-1.5%), and contained hexose sugars. Free-boundary electrophoretic patterns and sedimentation constants for the above fractions were measured. Their data showed that the four minor-protein fractions prepared from skimmilk were heterogeneous systems and that a major electrophoretic and ultracentrifugal peak

appeared to be a common component in these fractions. Whether the different procedures for preparing the minor-protein fractions yielded one or more identical components has not been elucilated.

Thus, from the above discussions, it is apparent that the proteose-peptone fraction is a group of heterogeneous proteins, composed essentially of three major components. However, to date, no individual components of the proteose-peptone fraction have been isolated and characterized. Also, since proteose-peptone has been reported to be obtained from the casein system (Jenness, 1959), as well as from whey, it would seem appropriate, in studying the distribution of proteose-peptone components, particularly component 3, to investigate their occurrence both in the casein micelle and the whey.

The presence of carbohydrate in protein has been recognized for years (Gottschalk, 1966). The association of carbohydrate with protein may be weak, as in the form of dissociable ion binding of mucopolysaccharides to protein, or it may be tightly bound in the form of a covalent linkage. A glycoprotein is defined as a protein-carbohydrate complex in which the carbohydrate is covalently bonded to the polypeptide and can only be dissociated by destructive methods such as acid hydrolysis. The carbohydrate portion contains amino sugars such as glucosamine and galactosamine, sialic acid and hexoses such as galactose, mannose and/or fucose. Some well known glycoproteins that have been

studied are egg ablumin (Johansen et al., 1961), ovomucoid (Chartterjee and Montgomer, 1962), α_1-acid glycoprotein (Eylar and Jeanloz, 1962), submaxillary mucoproteins (Graham and Gottschalk, 1960) and others. Glycoproteins have been found in milk proteins (Hipp et. al., 1961; Jolles et. al., 1962; Gordon et. al., 1963; Thompson and Brunner, 1959). Kappa casein is the main glycoprotein of the casein micelle and contains about 5% carbohydrate (Alais and Jolles, 1961). In whey proteins, the immune globulins, i.e., englobulin and pseudoglobulin are known to contain amino sugars and sialic acid (Smith et. al., 1946). That proteose-peptone of milk may be a group of glycoproteins is not surprising in view of its low nitrogen content. That it falls into the definition of a glycoprotein would also seem valid since in the course of its preparation, high salt concentration, i.e., salting out with ammonium sulfate, is employed which may have caused dissociation of the ionic binding between carbohydrate and protein, but would not affect the covalent linkage in a glycoprotein.

There are generally two principal types of covalent linkage between carbohydrate and polypeptide. One type involves the glycosidic linkage between the hydroxyl groups of threonine and serine of the polypeptide chain and the hexose or hexosamine of the carbohydrate moiety. This type of binding is alkali labile by the mechanism of β-elimination with the resultant cleavage of carbohydrate from protein (Neuberger et. al., 1966). Hence, in the course of

preparation of a glycoprotein, care should be taken to avoid prolonged exposure to alkaline pH. If an alkaline condition is achieved, one should check the protein for its carbohydrate content before and after treatment. Another type of linkage involves the N-acylglycosylamine bond between the amide nitrogen of asparagine or glutamine and hexose. This type of linkage is alkali stable. With regards to the sequence of carbohydrate moiety, it has generally been known that sialic acid occupies the terminal position in the carbohydrate chain, and that either hexose or hexosamine is linked to the protein moiety (Gottschalk, 1966).

Thompson and Brunner (1959) in their study of the carbohydrates of some minor protein of cow's milk, reported the concentrations of hexose, hexosamine, fucose and sialic acid in the proteose-peptone fraction and Weinstein fraction. Further, they indicated the presence of galactose, mannose, fucose, galactosamine (weak) and glucosamine in the proteose-peptone fraction. However, since individual components of the proteose-peptone fraction have not been isolated, no relative concentrations of carbohydrate in the various components of proteose-peptone have, so far, been studied.

PART I

THE PREPARATION AND ISOLATION OF COMPONENT 3 FROM HEATED AND UNHEATED SKIMMILK

INTRODUCTION TO PART I

The preparation of proteose-peptone was described previously (Thompson and Brunner, 1961). Our aim in devising a somewhat similar procedure was to obtain an enriched fraction of component 3 suitable for further purification. Techniques tried included fractionation with ethanol, pH adjustment and salting-out with ammonium sulfate. Ammonium sulfate fractionation was, by far, the most satisfactory method for obtaining enriched component 3. In this procedure, ammonium sulfate was added at various concentrations to heated whey at pH 4.6 following the removal of casein and heat-denatured whey proteins.

In the preparation of component 3 from proteose-peptone, it has been implied that component 3 from heated skimmilk was present mainly in the whey protein fraction since casein was removed at pH 4.6 after heat treatment. Hence, our initial step in locating component 3 from unheated skimmilk would be to fractionate the whey proteins. These fractions were examined by gel electrophoresis, together with the classical proteose-peptone, to see if a band corresponding in mobility to that of component 3 of proteose-peptone appeared. If such a band appeared in any of the whey protein fractions, its relative mobilities both before and after heat treatment were compared.

Once enriched component 3 from both heated and unheated skimmilk was obtained, several techniques seemed to offer possibilities for its purification. These included diethylaminoethyl (DEAE) cellolose and carboxymethyl cellulose or

CMC ion exchange chromatography, Sephadex or Bio-Rad gel filtration, preparative-gel electrophoresis and others. DEAE ion exchange chromatography and Bio-Rad gel filtration were tried with some degree of success, though a satisfactory isolation would require refinement of the techniques. Previously, the author had some experience in preparative-gel electrophoresis as a means of obtaining a highly purified casein component (Ng and Brunner, 1966). The analytical gel electrophoretic run of the enriched component 3 showed that component 3 was well separated from the other proteose-peptone components. Hence, the method of preparative-gel electrophoresis seemed to present the best hope for obtaining small amounts of electrophoretically homogeneous component 3.

However, certain possible shortcomings of the technique need to be considered. The major concern here is the possible cleavage of the carbohydrate moiety from glycoprotein when exposed to solution of alkaline pH. For satisfactory separation, the preparative acrylamide gel electrophoresis was usually conducted at buffer and gel pH values of 8.6 for a period of 10 to 14 hours at $< 10^{\circ}$ C. During such treatment, the type of carbohydrate-protein linkage known to be labile to alkali hydrolysis, i.e., the threonine or serine type of polypeptide linkage to carbohydrate might have occurred. Though, at present, we do not know the type of protein-carbohydrate linkage in proteose-peptone. Consequently, it seemed necessary to check the effect of the buffer pH exposure on the carbohydrate content of enriched

component 3. Once this question was answered, the technique could be used with some degree of confidence. The preparation isolated from the gel contained monomeric species of acrylamide which should be removed before any analysis was performed on the fraction. A convenient method of removing acrylamide from the extracted component 3 was by salting-out with ammonium sulfate, coupled with pH adjustment. The procedure was repeated several times to obtain component 3 essentially free of acrylamide.

Component 3 has been obtained from the whey protein fraction. However, we do not know as yet if part of the component 3 may be associated with micellar casein. Jenness (1959) reported the isolation of an enriched milk component-5 which was associated with the casein micelles in the preparation. Hence, it seems appropriate to investigate the distribution of proteose-peptone components, specifically component 3, in both casein and whey fractions. Two procedures of separating casein from whey proteins were used. One method was by preparative ultracentrifugation, which removed casein micelles in the form of a pellet, leaving whey proteins and the soluble casein in the supernatant. The other method was by isoelectric precipitation at pH 4.6, in which the casein, micellar as well as soluble, was removed, leaving the whey proteins in the supernatant. The four fractions, i.e., ultracentrifuged casein micelles and ultracentrifuged "whey" supernatant, isoelectric precipitated casein and whey supernatant were analyzed for the distribution of proteose-peptone components by gel electrophoresis.

EXPERIMENTAL METHODS

Apparatus

Raw milk was obtained from the university herds, both of Holstein and Jersey breeds, in ten gallon stainless steel cans. The raw milk was separated in a De-Laval, disc-type separator. Heating was conducted in an autoclave made by American Sterlizer Company. A Beckman Zeromatic pH meter equipped with glass electrode was used to measure pH values. Low-speed centrifugation (1000 x G) was performed in a model IV International centrifuge. Celite filter aid on a Buchner funnel was used for clarification of the supernatant when required. Dialyzing was done in Visking tubing obtained from Union Carbide Company. Intermediate-speed centrifugation (20,000 x G) was performed in a Servall (Type SS-1) centrifuge. Spectrophotometric measurements were made with a Cenco-Sheard-Sanford photelometer equipped with filters transmitting light at appropriate wavelengths. The horizontal gel electrophoretic cell was made from Plexiglass. A typical preparative cell had a dimension of 30 x 50 cm, with a gel bed thickness of about one cm (Figure 9). The buffer compartments held approximately one liter of buffer each and were fitted with 26-gauge platinum wire electrodes. A high-voltage power pack for preparative gel run was obtained from Savant Instrument Company (Range: 0-500 ma, 0-5,000 volts). The electrolytic destainer was constructed from Plexiglass, and was fitted with stainless steel plate electrodes. A

battery charger with a range of 12 volts and operated at about 3 ma was used as power pack for the destainer. Ultracentrifugation was performed in a Beckman Model L Preparative Ultracentrifuge.

Chemicals and Buffers

Chemicals and their suppliers are given as follows: ammonium sulfate (certified reagent) from Fisher Scientific Company; Cyanogum-41 gelling agent from E-C Apparatus Corporation; N, N, N′, N′-tetramethylethylenediamine (TMED) from Eastman Organic Chemical; ammonium persulfate (reagent grade) from Baker Chemical Company; starch-hydrolyzed for gel electrophoresis from Connaught Medical Research Laboratory; urea (reagent grade) from Baker and Adamson; boric acid (reagent grade) from Fisher Scientific Company; Tris (trimethylhydroxymethan, primary standard) from Sigma Chemical Company; citric acid (monohydrate, reagent grade) from Fisher Scientific Company; Folin-Ciocalteu reagent from Fisher Scientific Company; cupric sulfate ($CuSO_4 \cdot 5H_2O$, certified reagent) from Fisher Scientific Company; potasium tartrate (certified reagent) from Fisher Scientific Company; orcinol (monohydrate, reagent grade) from Fisher Scientific Company.

The following buffer preparations are given in the Appendix: discontinuous buffer systems used in analytical and preparative acrylamide gel electrophoresis; discontinuous buffer systems used in starch-urea gel electrophoresis;

buffers used in checking the effect of buffer pH exposure on the hexose content of purified component 3; Folin-Ciocalteau reagents and buffers; orcinol-sulfuric acid reagents.

Preparatory Procedure

Procedure for Obtaining Enriched Component 3 from Heated Skimmilk

Whole raw milk obtained from the university herd was maintained at temperatures between 35° to 40° C until separated. The skimmilk was placed into several 5-liter stainless steel beakers and covered with Aluminum foil. Heating was conducted in an autoclave, with steam pressure of less than 5 psi, at a temperature slightly above 100° C, for a period of 30 minutes. The heated skimmilk was immediately cooled with running tap water, and allowed to come to room temperature. Casein and denatured whey proteins were precipitated by adjusting the pH to 4.6 with 1 N HCl. The precipitate was removed by centrifugation at 1,000 x G for 10 minutes in the International centrifuge. On occasion, the supernatant remained turbid after centrifuging, necessitating filtration through a Buckner funnel covered with Celite filter aid. The clear supernatant contained the proteose-peptone components. Fractionation with ammonium sulfate was carried out at pH 4.6. Solid ammonium sulfate was added to 35% saturation. The solution was left at room temperature for 30 minutes to allow the precipitate to form

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and settle. The solution was centrifuged at 1,000 x G for 20 minutes. The precipitate was redispersed in deionized water, pH adjusted to 6.5, dialyzed under several changes of deionized water and freeze dried. Then, more ammonium sulfate was added to the 35% saturated $(NH_4)_2SO_4$ supernatant until another precipitate was formed at 55% saturation. The solution was centrifuged at 1,000 x G for 20 minutes. The precipitate was treated as before. The 55% saturated $(NH_4)_2SO_4$ supernatant was made to 80% saturation and another precipitate was formed. The final precipitate was redispersed as before.

Procedure for Obtaining Enriched Component 3 from Unheated Skimmilk

Whole raw milk was separated at 35° to 40° C. The skimmilk was acidified with 1 N HCl to pH 4.6. The precipitated casein was filtered on cheese cloth, and the whey filtered through a Buchner funnel covered with Celite filter aid. The pH of the clear whey was adjusted to 6.5 with 1 N NaOH. Solid ammonium sulfate was added to whey to 50% saturation. The precipitate containing the crude globulin fraction was removed by centrifugation at 1,000 x G for 20 minutes and saved for further working. The supernatant, designated as S_1 (Figure 2), containing the crude albumin fraction, was discarded. The precipitate was redissolved in distilled water to about 3% protein concentration, and the solution was adjusted to pH 4.6. Ammonium sulfate was

added to 25% saturation. The precipitate, P_1, was removed by centrifugation at 1,000 x G for 30 minutes. The supernatant was adjusted to pH 6.0 with 1 N NaOH and ammonium sulfate was added to 40% saturation. The solution was held at 37° C for 30 minutes. The precipitate, P_2, was removed by centrifugation at 1,000 x G for 20 minutes. More ammonium sulfate was added to the supernatant bringing the salt concentration to 45% saturation. The solution was held at 37° C for one hour and was centrifuged at 1,000 x G for 20 minutes. The final precipitate was redissolved in water, the solution was adjusted to pH 6.5, dialyzed and freeze dried. To check the heat stability of the fraction, a small amount of the supernatant (after removal of P_2 precipitate) was dialyzed against deionized water to remove most of the salt, and was pervaporated to the same original volume before dialysis. The solution was heated at 100° C for 30 minutes and cooled to room temperature. The pH was adjusted to 4.6 followed by centrifugation at 1,000 x G for 20 minutes. The clear supernatant was adjusted to pH 6.5, dialyzed and freeze-dried.

Procedure for Checking the Effect of Gel Buffer pH on the Carbohydrate Content of the Purified Component 3 Preparation

The buffer system used for the preparative-acrylamide gel electrophoresis was a discontinuous one, with borate buffer (pH 8.6) in the electrode compartments and Tris-citrate buffer (pH 8.6) in the gel. To assess the effect

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of buffer pH on the carbohydrate content of purified component 3, buffers ranging in pH from 4.6 to 8.6 were employed. The buffer preparations are described in the Appendix, Part I. Purified component 3 (heated preparation) was dissolved and exposed in the buffers for a period of 12 hours at room temperatures. Then, the pH of the buffers were adjusted to 4.6. Solid ammonium sulfate was added to 80% saturation to salt-out the protein which was collected by centrifugation at 20,000 x G for 15 minutes in a Servall (Type SS-1) centrifuge. The precipitate was redissolved in deionized water and the solution was adjusted to pH 7.0, dialyzed and freeze dried. The treated, purified component 3 preparations were analyzed for total hexose by the orcinol-sulfuric acid procedure (Winzler, 1955). The preparation of the orcinol-sulfuric acid reagents is given in the Appendix, Part I. Two to ten milligrams of the buffer-exposed component 3 samples were dissolved in one ml of 0.1 N NaOH. A blank (one ml of water) was prepared. Eight and one half milliliters of the orcinol-sulfuric acid reagent was added to the tube and the solution mixed by inversion. The tube was capped with a glass marble to minimize evaporation and placed in a water bath at 80° C for 15 minutes. The tube was cooled in tap water and readings (% transmission) were taken in a Cenco-Sheard-Sanford photelometer equipped with a filter transmitting light at 540 mμ.

Isolation Procedure for Component 3 from the Enriched Fraction by Preparative Acylamide-Gel Electrophoresis

Both continuous and discontinuous buffer systems can be used for gel electrophoresis. However, the discontinuous system has the advantage of showing a sharp visible, frontal band, enabling one to follow the progress of electrophoresis and hence was adopted for this purpose. The buffer preparations, Tris-citrate buffer in the gel and borate buffer in the electrode compartment, were described in Appendix, Part I. For the gel buffer a 1:10 dilution of the stock Tris-citrate solution (pH 8.6 to 8.9) was used. For the electrode buffer, two parts of stock borate to three parts of water, at pH 8.6, was used. Cyanogum-41 gelling agent was dissolved in the Tris-citrate buffer to make an 8% acrylamide solution (w/v). This solution was filtered to remove residual insoluble particles. N, N, N, N-tetramethylenediamine (0.1 ml/100 ml gel solution) was added to the clear gel solution, followed by the addition of ammonium persulfate (0.2 gm/100 ml gel solution) with constant stirring. The solution was poured immediately into the preparative gel bed and a continuous slot former was positioned approximately 3 cm from the cathodic end of the bed. Electrical contact between the buffer and gel was provided by means of one-quarter-inch holes in the gel/buffer partition. The holes were sealed temporarily with electrical insulation tape placed on the buffer side. This strip of tape was removed

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after the gel formed. A plastic cover, placed over the cell and flushed with nitrogen, provided the necessary low-oxygen atmosphere required for the gelling agent to polymerize. Gelling was completed in one minute but the gel was allowed to stand for 20 minutes before using. Cold water, circulating under the gel bed, served to dissipate the heat generated during the run. A 5 ml portion of a 5% component 3 (enriched) solution (250 mg of protein in borate buffer) was added to the sample slot. The entire assembly was covered with Saran wrap to retard loss of moisture. A high-voltage power supply provided the current flow with a current density of from 8 ma to 10 ma per cm^2 of gel cross-sectional area. Electrophoresis was conducted for a period of from 10 to 12 hours during which time the current dropped to 3 ma/cm^2. No attempt was made to maintain constant current during the run.

At the end of the electrophoretic run, 2-cm wide strips were removed from each side of the gel bed, stained with amido black and destained electrophoretically for about one hour to locate the position of the migrating zone of component 3. The stained sections were replaced to their original positions in the gel and a cross-sectional strip corresponding to the component 3 zone (unstained portion) was removed in preparation for the protein extraction step. The excised gel was macerated in a Waring blender with deionized water to make a gel slurry. More water was added to the gel slurry and the pH was adjusted to 5.0 with 1 N

HCl. The diluted gel slurry was stirred in the cold for one day to allow diffusion of protein from gel particles. The equilibrated gel slurry was centrifuged at 1,000 x G for 20 minutes to separate the gel particles from the aqueous phase. The supernatant was saved. The gel particles were again extracted twice with deionized water. The supernatants from the three extractions were combined, adjusted to pH 6.5, dialyzed, pervaporated, and freeze-dried.

Since monomeric species of the gelling agent were recovered with the protein in the extracted solution, additional steps were taken to recover gel-free protein. For component 3, salting-out with sodium chloride, coupled with pH adjustment, was used. The procedure was as follows: Extracted component 3 was dissolved in deionized water to 1% solution. Solid sodium chloride was added to the solution until a saturation concentration was reached (36 gm NaCl/100 ml solution). The pH of the solution was adjusted to 2.0. A flocculent precipitate was observed which was collected by centrifugation at 20,000 x G for 15 minutes. The precipitate was again redissolved in deionized water to a concentration of approximately one percent. The solution was saturated with sodium chloride and the pH adjusted to 2.0. Again, a flocculent precipitate was obtained. This process was repeated two to four more times to insure the complete removal of acrylamide. To determine the number of precipitation cycles required for effective removal of acrylamide, a colorimetric Folin-Ciocalteu procedure for "total

protein" content was performed on the precipitate obtained from the four precipitation cycles. The preparation of Folin-Ciocalteu reagents was given in Appendix, Part I. The colorimetric procedure was as follows: Five millliliters of the mixed sodium carbonate-potasium tartrate reagents was added to one milliliter of sample solution containing 0.5 mg of isolated component 3. The solution was allowed to stand at room temperature for 10 minutes prior to adding 0.5 ml of Folin-Ciocalteu reagent. This solution was allowed to stand for 30 minutes before making transmission readings at 525 mμ with a Cenco-Sheard-Sanford photelometer.

Distribution of Component 3 in Casein and Whey Protein Fractions

Proteose-peptone components were obtained from casein and whey protein fractions by ultracentrifugation and isoelectric point precipitation as depicted in the following sections.

Ultracentrifugation

The preparative scheme is shown in Figure 5. Fresh raw milk was separated at 40° C in a De-Laval cream separator. The skimmilk phase was ready for ultracentrifugation. Ultracentrifugation was performed in a Beckman Model L preparative ultracentrifuge, equipped with a type 50 rotor, run at a speed of 48,000 rpm for 90 minutes at room temperature. The casein pellet was dispersed in deionized water with

stirring and was made up to the original skimmilk volume. Both ultracentrifuged casein and ultracentrifuged "whey" supernatant were heat treated at 100° C for 30 minutes in an autoclave. After cooling to room temperature, the pH of the solution was adjusted to 4.6 with 1 N HCl. The solutions were centrifuged at 1,000 x G for 20 minutes. The supernatants of both fractions, containing the proteose-peptone components, were adjusted to pH 6.5 with 1 N NaOH, dialyzed and freeze-dried.

Isoelectric Precipitation

The preparative scheme is shown in Figure 6. Skimmilk was adjusted to pH 4.6 with 1 N HCl. The solution was centrifuged at 1,000 x G for 15 minutes. The whey supernatant was adjusted to pH 6.7 before heat treatment. The isoelectric casein was redissolved in deionized water, with a small addition of 1 N NaOH, and made up to original skimmilk volume. Since small amount of whey protein may be occluded in the first casein precipitate, another IEP precipitation was undertaken. The casein solution was adjusted to pH 4.6. The solution was centrifuged at 1,000 x G for 20 minutes. The precipitated casein, which was almost free of whey protein, was redissolved in deionized water with small addition of 1 N NaOH. The casein solution was adjusted to pH 6.7 and made up to original volume of skimmilk. The whey and the twice IEP precipitated casein solution were heat treated at 100° C for 30 minutes in an autoclave. After

cooling to room temperature, the solutions were adjusted to pH 4.6 with 1 N HCl. The solutions were centrifuged at 1,000 x G for 20 minutes. The supernatants from the two fractions, containing the proteose-peptone components, were adjusted to pH 6.5, dialyzed and freeze-dried.

RESULTS AND DISCUSSIONS

Preparation of Enriched Component 3 from Heated and Unheated Skimmilk

Three protein fractions from the proteose-peptone fraction were obtained by the ammonium sulfate fractionation procedure when applied to heated skimmilk as outlined in Figure 1. Acrylamide gel electrophoretic patterns of the three fractions and the classical proteose-peptone fraction are shown in Figure 7. The preparation of the classical proteose-peptone is outlined in the Appendix, Part I. Gel electrophoretic patterns of these fractions revealed some interesting observations. Evidently, enrichment of certain components of proteose-peptone was obtained in the various ammonium sulfate cuts. The proteose-peptone fraction corresponding to the 35% saturated ammonium sulfate cut shows an enrichment of component 5; the 55% saturated ammonium sulfate cut, an enrichment of component 3; and the 80% saturated ammonium sulfate cut, an enrichment of component 8. Hence, the enriched component 3 fraction corresponding to the 55% saturated ammonium sulfate cut was used as starting material for the isolation of component 3.

To locate component 3 in unheated skimmilk, whey was fractionated as outlined in Figure 2. The various protein fractions, designated S_1, P_1, P_2, and P_3, were examined electrophoretically in acrylamide gels, together with the proteose-peptone fraction (see Figure 8A). As observed,

the P_3 fraction contained a component similar in mobility to component 3 of the proteose-peptone fraction. The P_3 fraction was heated to 100° C for 30 minutes as a check on heat alteration. The electrophoretic pattern of the heated P_3 fraction showed a component similar in electrophoretic characteristics to component 3 in proteose-peptone (Figure 8B). Thus, the component in the P_3 fraction appeared to be component 3 in unheated skimmilk. The gel electrophoretic patterns of enriched component 3 from heated and unheated skimmilk is shown in Figure 8C. Judging from the concentration of component 3 relative to other proteose-peptone components as they appeared in the gel electrophoretogram, the enrichment procedures can be considered satisfactory.

Effect of Gel Buffer pH on the Carbohydrate Content of the Purified Component 3 Preparation

The hexose contents of the glycoprotein that was exposed to buffers of various pH are shown in Table 1. Here, the hexose content was used as an index of the carbohydrate content of purified component 3. The colorimetric value or absorbance readings at 540 mμ was a measure of the hexose content. The hexose contents of purified component 3 exposed to neutral buffer (pH 7.0) and deionized water were used as "standards," with presumably no loss of hexose from the glycoprotein in these solutions. As observed from Figure 3, the purified glycoprotein that was exposed to the acidic and basic buffers (citrate-phosphate, pH 4.6; Tris-

citrate, pH 8.6; borate, pH 8.6) showed the same relative hexose contents as those of the glycoprotein exposed to the neutral buffer (Tris-citrate, pH 7.0) and deionized water. Thus, exposure to the buffers ranging in pH from 4.6 to 8.6 did not markedly affect the hexose content of the preparation. Exposure to 0.1 N NaOH (pH $>$ 12) decreased the hexose content slightly (Figure 3).

That the hexose contents of the glycoprotein exposed to buffers of various pH values remained essentially unchanged does not preclude the possibility that the glycoprotein might undergo physical alterations in molecular association during such exposures. Hence, the various buffer-exposed component 3 samples were run on acrylamide gels at pH 8.6 to check their electrophoretic characteristics. Again, the gel patterns of the treated, purified component 3 samples remained the same (Figure 9). Thus, it can be assumed that exposure to the buffer systems used in preparative acrylamide gel electrophoresis, i.e., Tris-citrate and borate buffer at pH 8.6, for the duration of the electrophoretic run at temperature less than 10° C, did not result in drastic cleavage of carbohydrate from the glycoprotein.

Isolation of Component 3 from the Enriched Fractions

Enriched component 3 samples were applied on the preparative acrylamide gels for the isolation of the electrophoretically homogeneous component. A typical electro-

phoretogram from the two strips of the preparative gel is shown in Figure 11. The resolution of enriched component 3 in the preparative acrylamide gel was quite satisfactory and comparable to the resolution obtained in analytical gel electrophoresis. Also, component 3 was well separated from the other proteose-peptone components and, hence, was conveniently removed from the gel.

Component 3 was extracted from the excised gels by the process of equilibrium-diffusion, using several changes of fresh, deionized water. The excised gels was macerated into a slurry to provide greater efficiency in extraction. The extracted component 3 contained monomeric species of the acrylamide gelling agents, which must be removed from the sample. The protein content of the extracted component 3 increased with subsequent removal of acrylamide by the salting-out technique (see Figure 4). Here, the "total protein" content was measured by the absorbance values obtained with the Folin-Ciocalteu reaction. Component 3 was essentially free of acrylamide after the second precipitation (Figure 4). Hence, three time precipitations were adopted for the removal of acrylamide. The procedure did not preclude the possibility that small amount of acrylamide may be tightly complexed with the protein and cannot be removed by further precipitation. The gel electrophoretic patterns of the isolated component 3, together with their enriched fractions, both from heated and unheated skimmilk, are shown in Figure 12A. The starch-urea gel electrophoretic

patterns of the isolated component 3 are shown in Figure 12B. The incorporation of urea in the gel increased the mobility of component 3. The isolated component 3 was essentially electrophoretically homogeneous and free of other proteose-peptone components.

Distribution of Component 3 in Casein and Whey

Proteose-peptone components were obtained from casein and whey by ultracentrifugation and isoelectric precipitation. The term proteose-peptone is applied to the protein fraction that remains in the supernatant after heat treatment and subsequent acid precipitation at pH 4.6. The gel electrophoretic patterns of proteose-peptone obtained from 1) ultracentrifuged "whey" supernatant; 2) ultracentrifuged casein micelle; 3) whey after isoelectric precipitation of skimmilk at pH 4.6 and 4) isoelectric (pH 4.6) precipitated casein, are shown in Figure 13. It is interesting to note that component 3 is present in the proteose-peptone fraction from whey, but is conspicuously absent in the proteose-peptone fraction from casein. This is rather interesting from the conjecture that if component 3 is associated with other proteose-peptone components, one would normally expect that part of it would also be found in the casein fraction, since the other proteose-peptone components appear to be present, in part, in the casein fraction. Yet the gel electrophoretic patterns show that component 3 is found only in the proteose-peptone obtained from whey. Previous isolations

of component 3 from unheated skimmilk showed that component 3 resides mainly in the classical lactoglobulin fraction of whey.

SUMMARY OF PART I

An ammonium sulfate fractionation procedure was developed for obtaining enriched component 3, both from heated and unheated skimmilk. Component 3 was isolated from the enriched fractions by a preparative acrylamide gel electrophoretic technique. The buffer system used in preparative acrylamide gel electrophoresis did not produce apparent cleavage of carbohydrate from the glycoprotein. The component 3 preparations, from heated and unheated skimmilk, were electrophoretically homogeneous and essentially free of other protein components. A study of the distribution of proteose-peptone components in casein and whey indicated that component 3 was present only in the whey and mainly in the lactoglobulin fraction.

TABLE 1

Colorimetric values for hexose content of purified component 3 exposed to buffers of various pH values as determined by the Orcinol-Sulfuric acid method (see Figure 3)

Buffer (ion strength = 0.1)	Sample Weight (mg/ml)	Absorbance (at 540 mμ)
Citrate-phosphate pH 4.6	2.2	0.218
	4.7	0.403
	6.7	0.582
Citrate-phosphate pH 7.0	2.6	0.252
	6.5	0.569
	9.2	0.796
Deionized water pH 6.5	2.0	0.197
	4.4	0.377
	8.5	0.745
Tris-citrate pH 8.6	3.1	0.310
	6.4	0.599
	9.1	0.796
Borate pH 8.6	2.3	0.280
	6.5	0.602
	8.5	0.788
0.1 N NaOH pH > 12	2.0	0.166
	5.0	0.393
	7.7	0.553

TABLE 2

Folin-Ciocalteu colorimetric values
for "total protein" content to determine
the number of precipitation steps required
for effective removal of acrylamide from protein extract
(see Figure 4)

Samples	Absorbance (at 525 mμ)
Extracted component 3* (contains acrylamide)	0.292
1st Precipitate	0.648
2nd Precipitate	0.721
3rd Precipitate	0.721
4th Precipitate	0.721

*Concentration of sample = 0.5 mg/ml

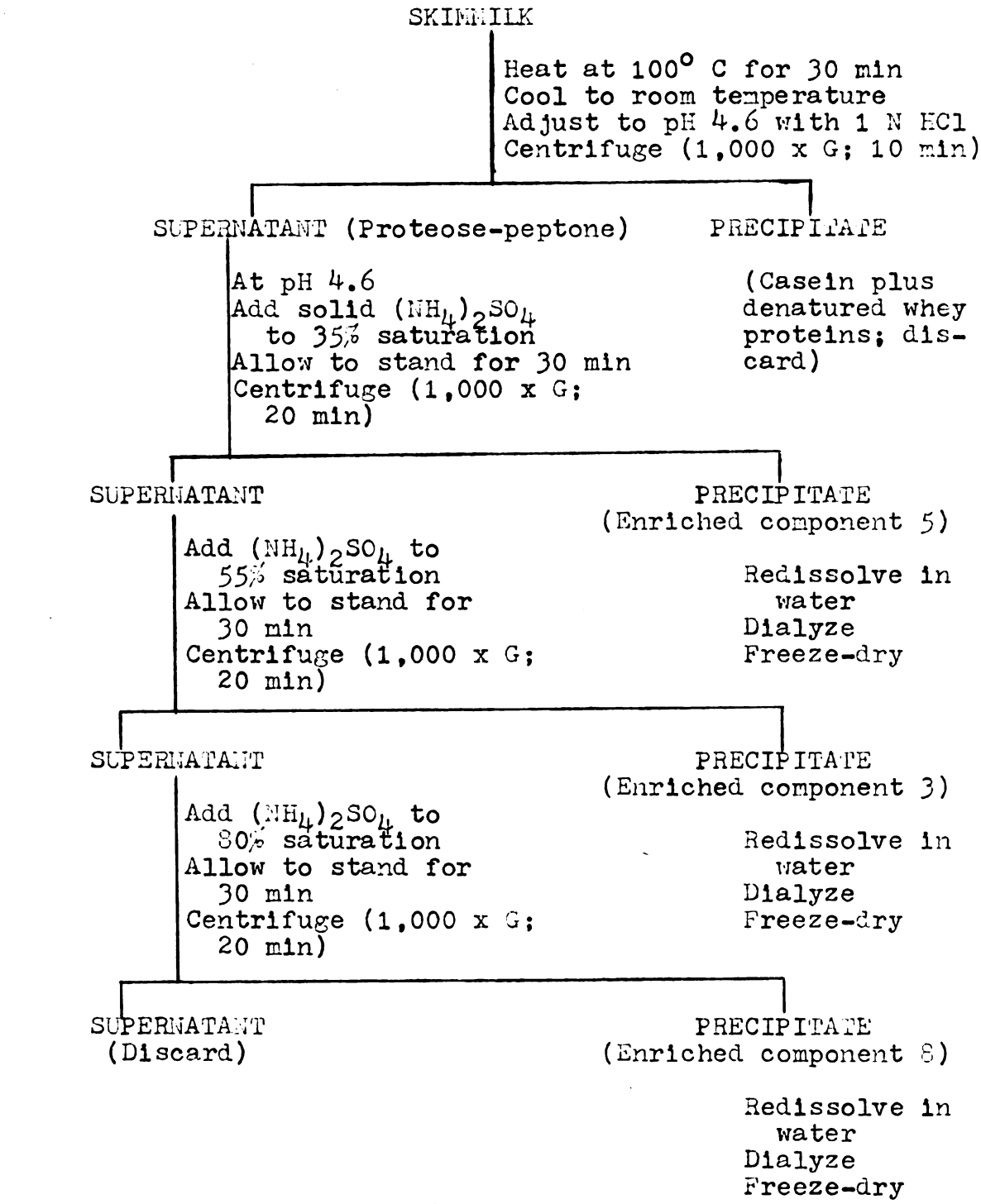
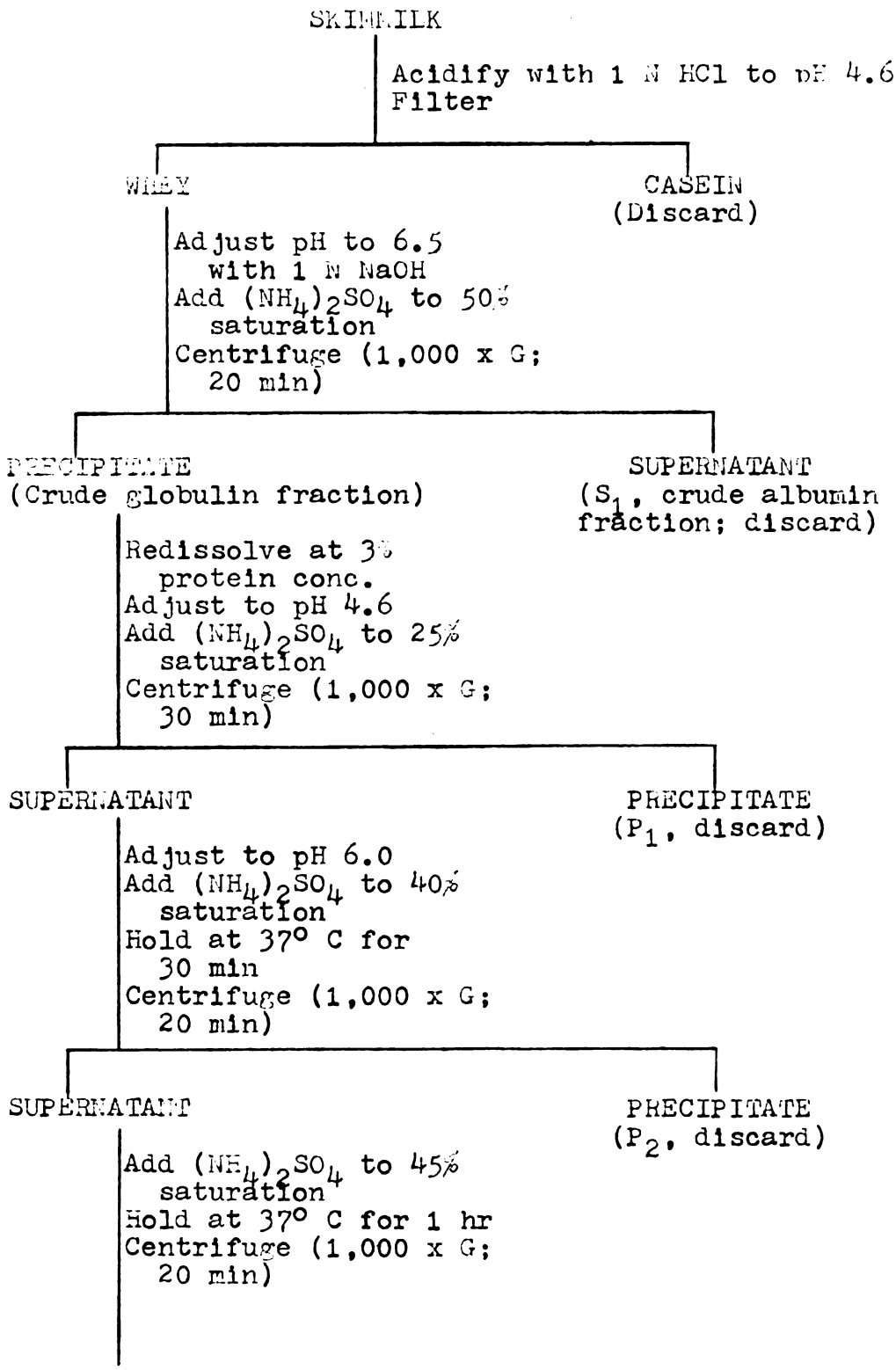


Figure 1. Procedure followed for obtaining enriched component 3 from heated skimmilk.



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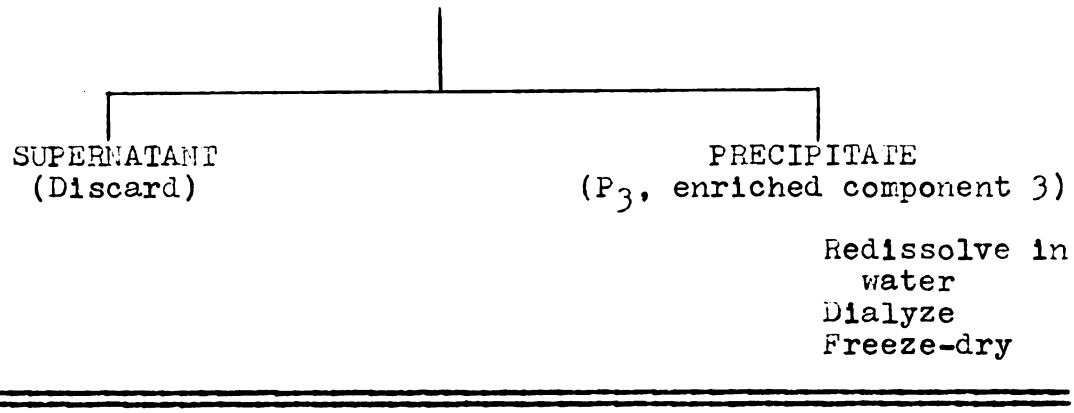


Figure 2. Procedure followed for obtaining enriched component 3 from unheated skimmilk.

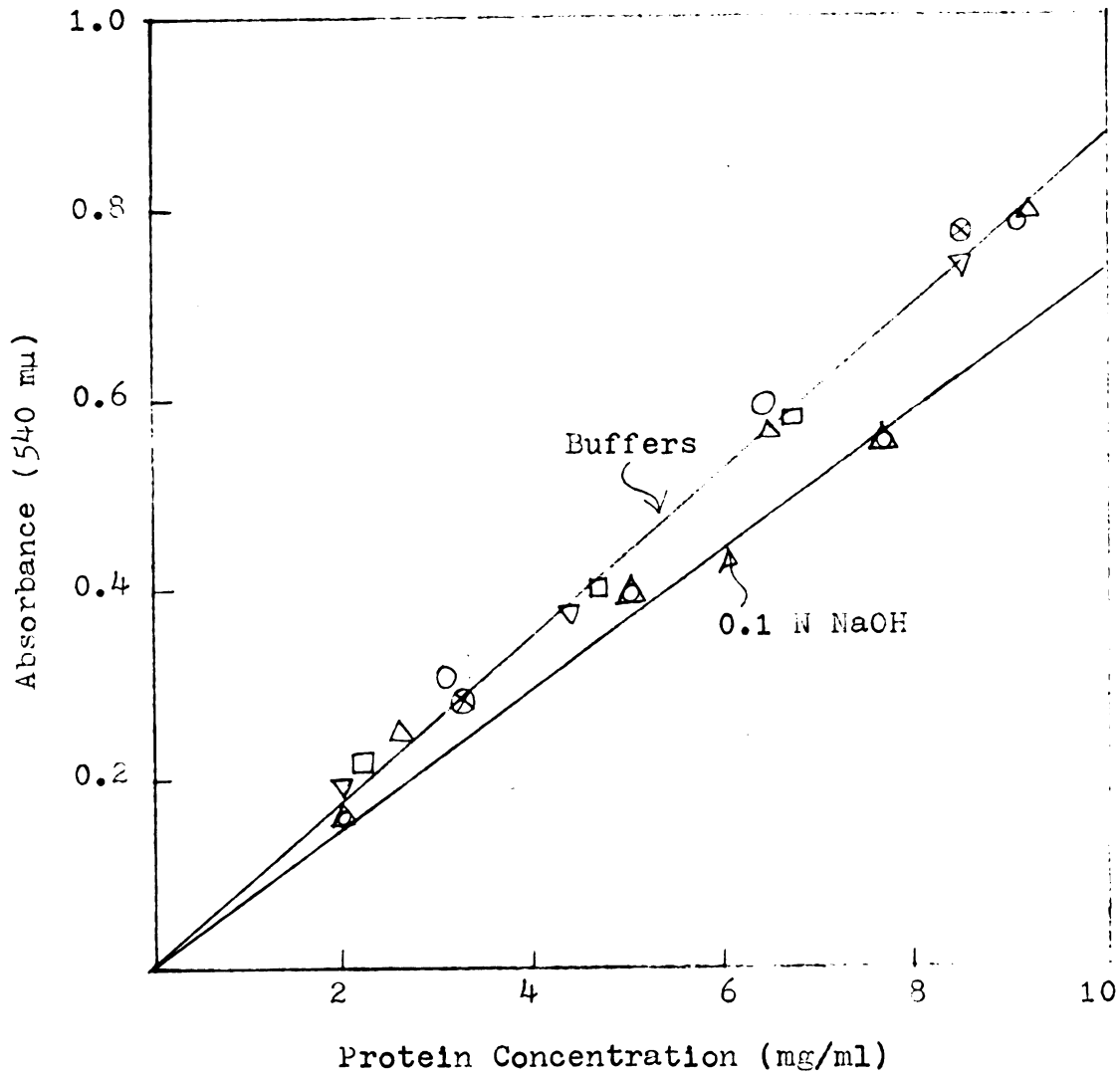


Figure 3. Colorimetric values (Orcinol-Sulfuric acid method) for hexose contents of purified component 3 exposed to buffers of various pH values. Legend: □ , citrate phosphate pH 4.6; △ , citrate phosphate pH 7.0; ▽ , deionized water; ○ , Tris-citrate pH 8.6; ⊗ , borate pH 8.6; ▲ , 0.1 N NaOH pH 12.

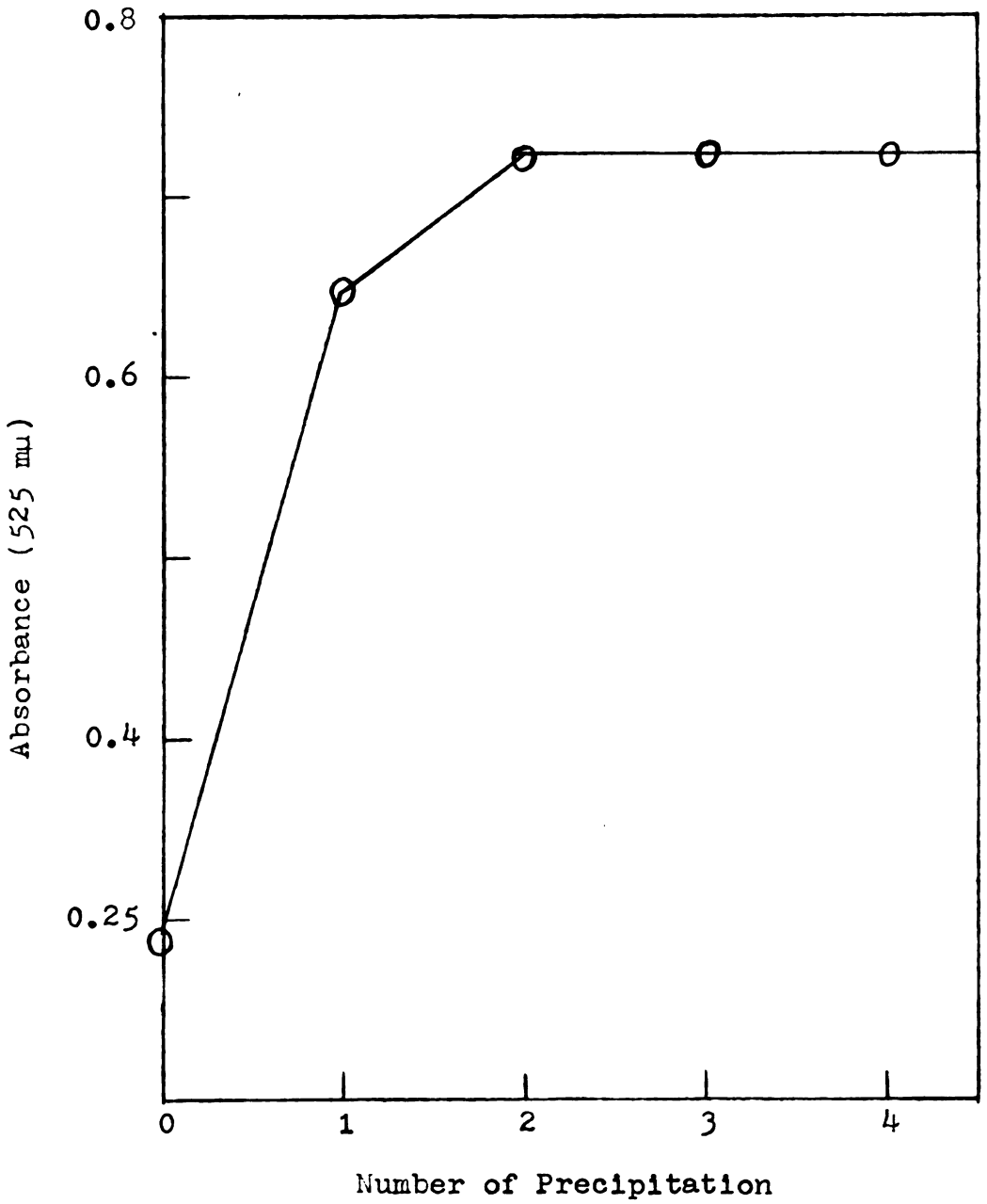


Figure 4. Folin-Ciocalteu colorimetric values for "total protein" content to determine the number of precipitation steps required for effective removal of acrylamide from protein extract.

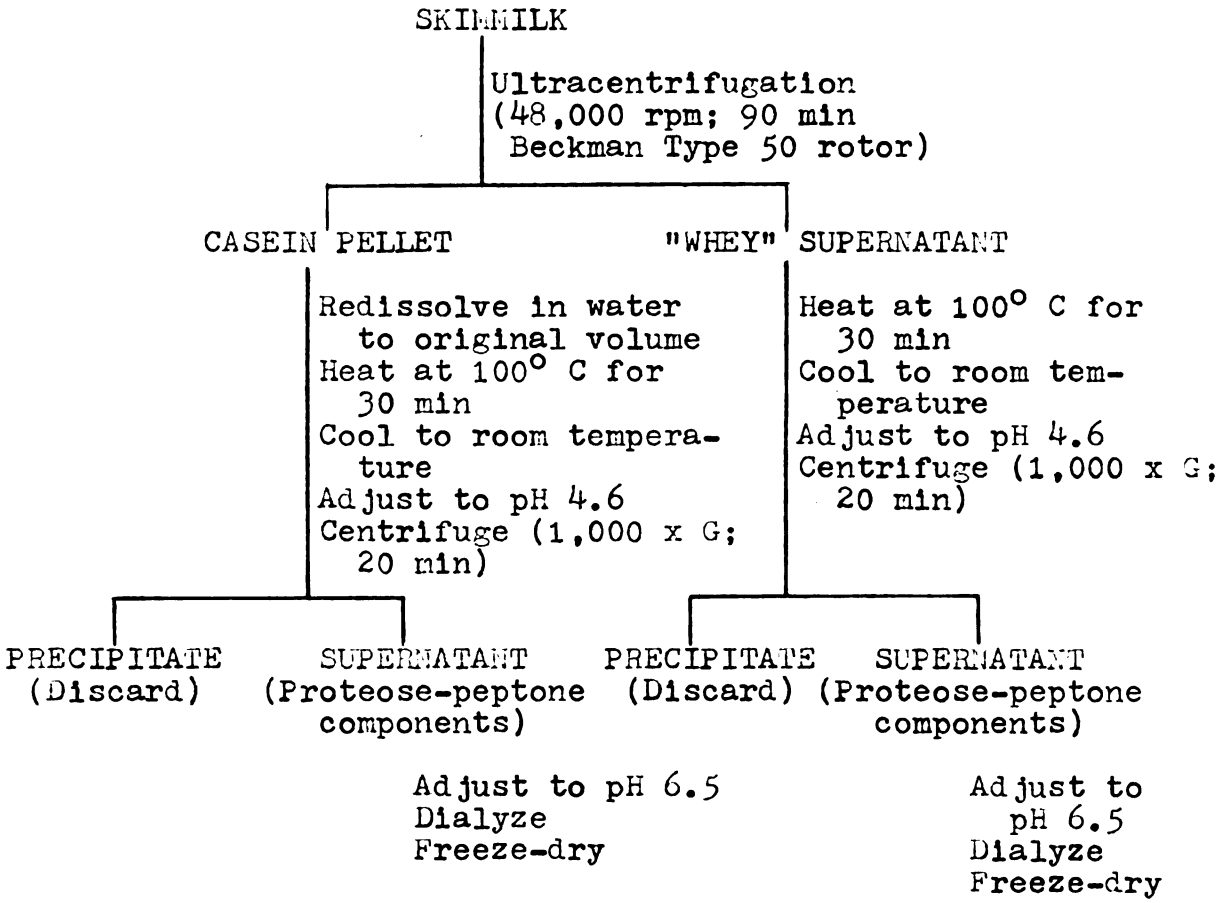


Figure 5. Procedure followed for obtaining proteose-peptone components from casein and whey by ultracentrifugation.

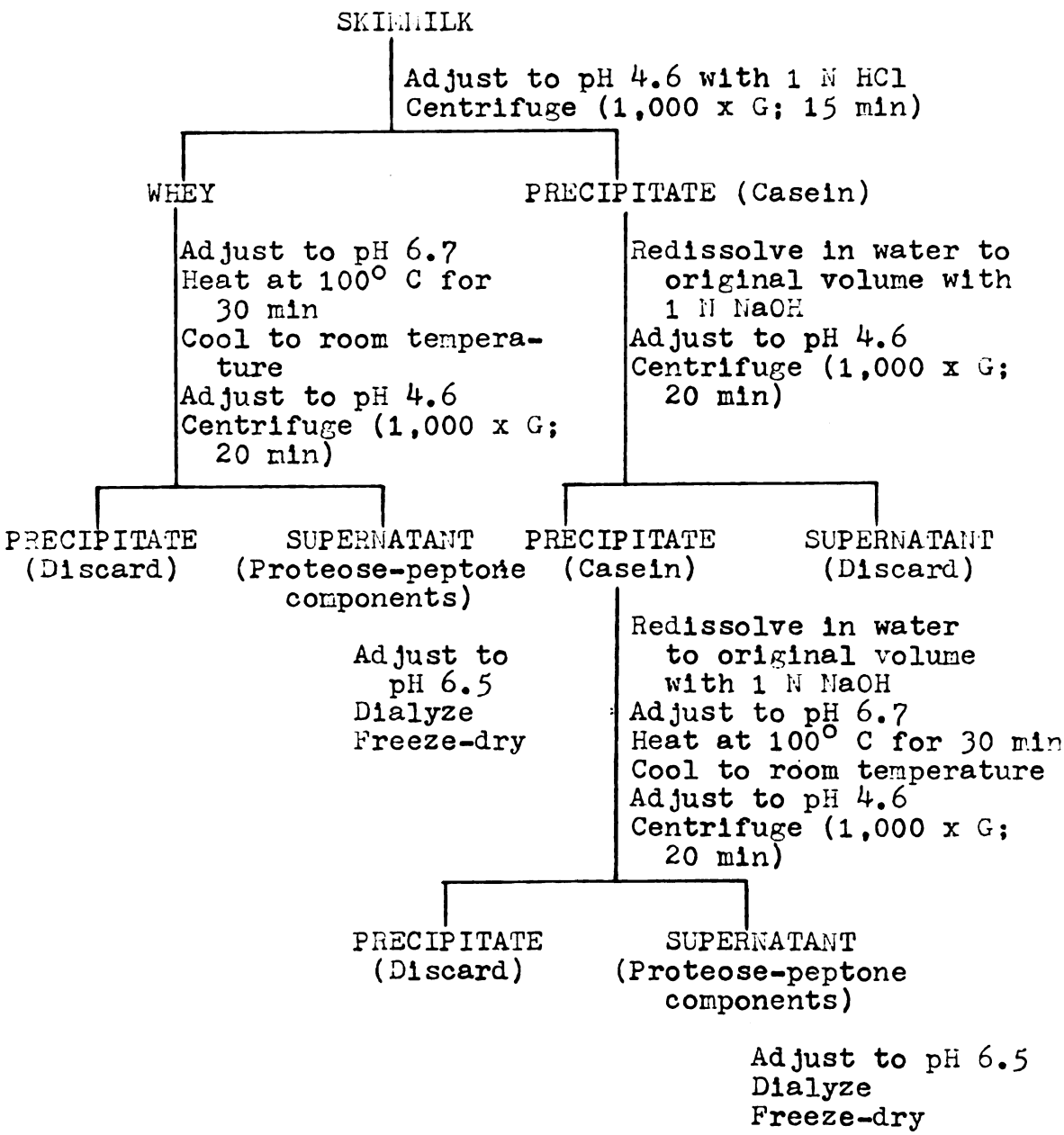


Figure 6. Procedure followed for obtaining proteose-peptone components from casein and whey by isoelectric precipitation at pH 4.6.

Figure 7. Acrylamide gel electrophoretic patterns of proteose-peptones obtained from the preparative scheme (Figure 1): 1) classical proteose-peptone (Appendix, Part I), 2) enriched component 5, 3) enriched component 3, 4) enriched component 8. Discontinuous buffer systems (Appendix, Part I).

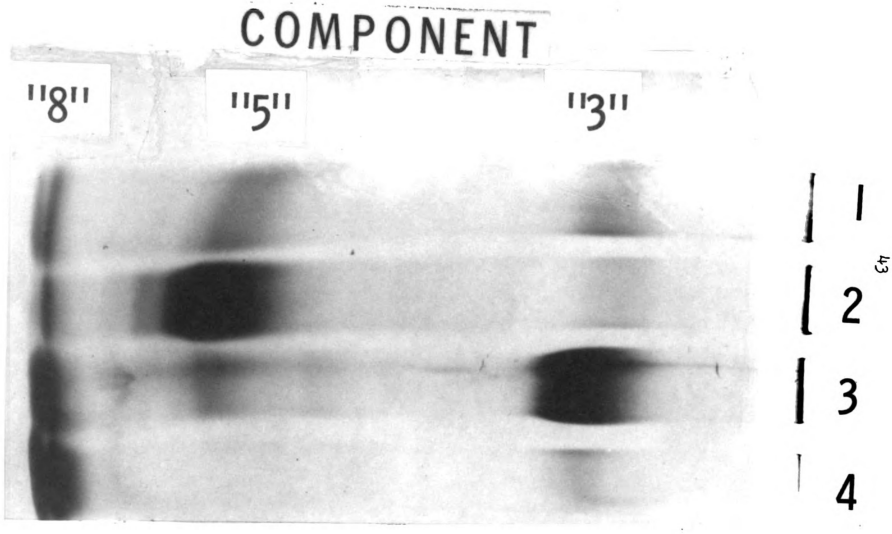
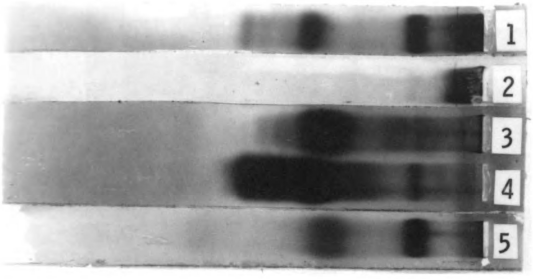

COMPONENT
"8"
"5"
"3"
1
2
3
4

Figure 8. Acrylamide gel electrophoretic patterns of proteose-peptone and various whey protein fractions as designated in the isolation scheme, Figure 2. Discontinuous buffer system (Appendix, Part I). Colume A: 1) P_3 fraction, 2) P_2 fraction, 3) P_1 fraction, 4) S_1 fraction, 5) proteose-peptone. Column B: 1) P_3 fraction following heat treatment, 2) P_3 fraction prior to heat treatment, 3) proteose-peptone. Column C: 1) proteose-peptone, 2) enriched component 3 fraction from the preparative procedure as outlined in Figure 1, 3) enriched component 3 fraction from the preparative procedure as outlined in Figure 2.


1
2
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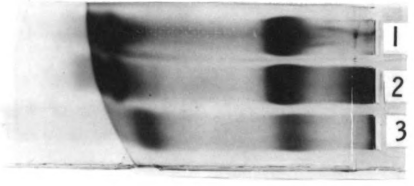
5
A


1
2
3
B


1
2
3
C

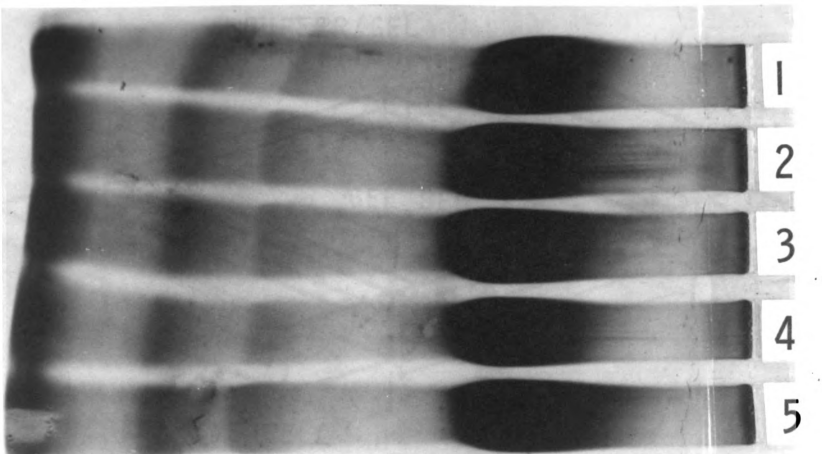


Figure 9. Acrylamide gel electrophoretic patterns of enriched component 3 (heated preparation) exposed to buffers of various pH for 12 hours: 1) citrate-phosphate (pH 4.6), 2) Tris-citrate (pH 8.6), 3) borate (pH 8.6), 4) deionized water (pH 6.5), 5) 0.1 N NaOH solution. Discontinuous buffer system (Appendix, Part I).

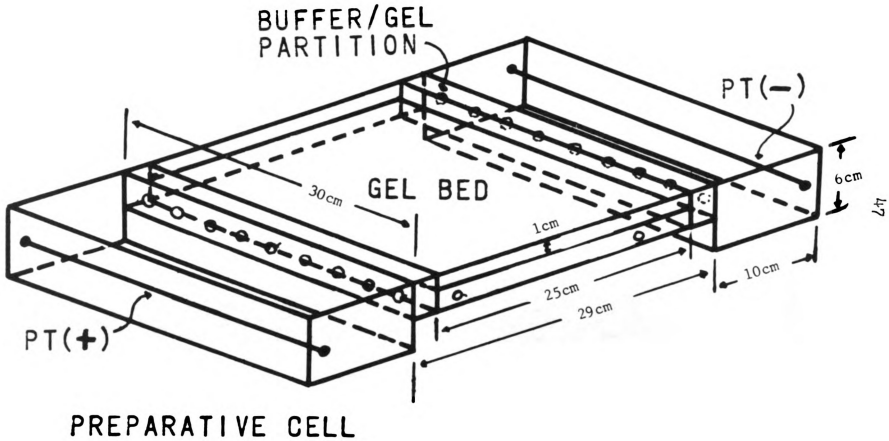


Figure 10. The preparative electrophoretic cell used in the isolation of component 3 from the enriched fraction.

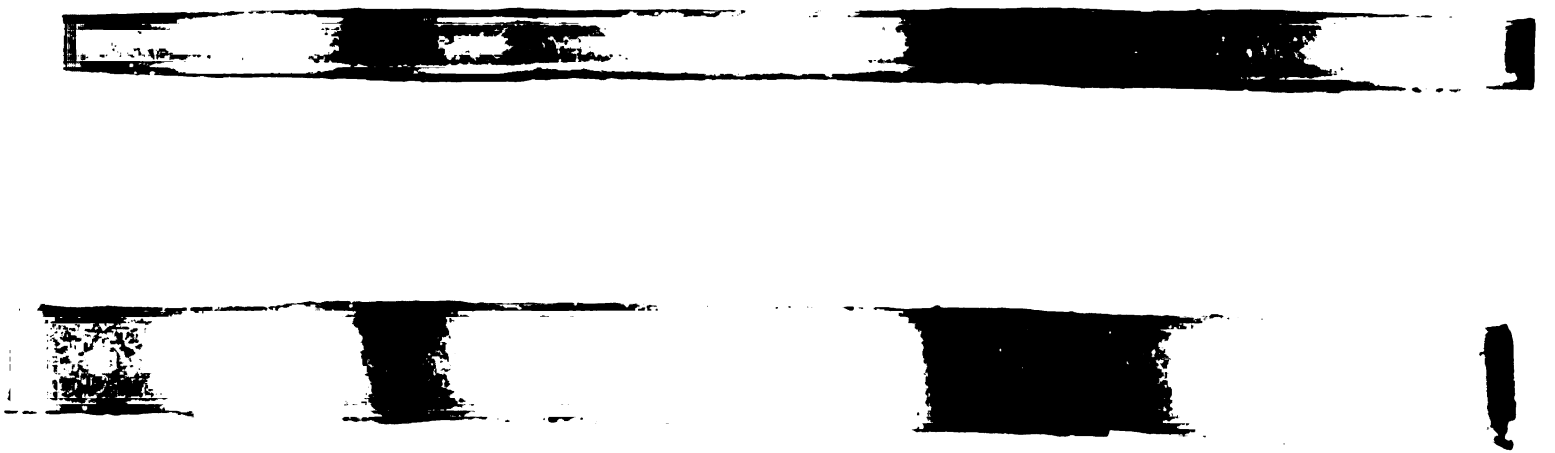
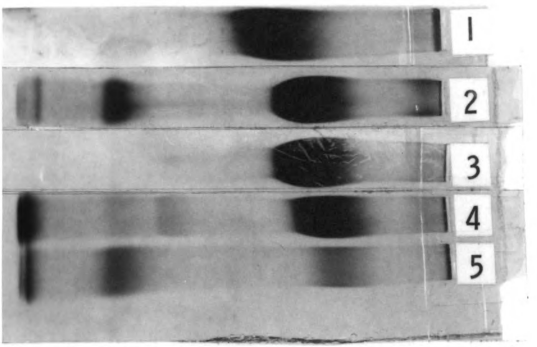
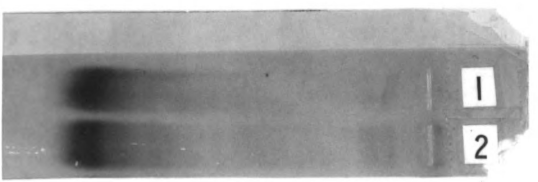


Figure 11. Acrylamide gel electrophoretic patterns of purified component 3 from two side strips of the preparative gel.

Figure 12. Acrylamide gel electrophoretic patterns of component 3 isolated from the preparative-scale acrylamide gel electrophoresis. Colume A. Acrylamide gel electrophoretic patterns of: 1) isolated component 3 (unheated preparation) obtained from the preparative gel, 2) enriched component 3 (unheated preparation) applied to the preparative gel, 3) isolated component 3 (heated preparation) obtained from the preparative gel, 4) enriched component 3 (heated preparation) applied to the preparative gel, 5) proteose-peptone. Discontinuous buffer system (Appendix, Part I). Colume B. Starch-urea gel electrophoretic patters of: 1) isolated component 3 (heated preparation), 2) isolated component 3 (unheated preparation). Discontinuous buffer system (Appendix, Part I0.



A



B

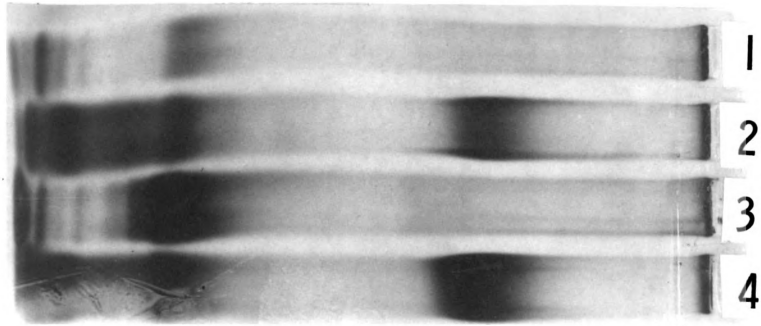


Figure 13. Acrylamide gel electrophoretic patterns of proteose-peptones obtained from: 1) heated IEP casein, 2) heated whey, 3) heated, ultracentrifuged casein, 4) heated, ultracentrifuged "whey." Discontinuous buffer system (Appendix, Part I).

PART II

THE CHEMICAL AND PHYSICAL PROPERTIES OF COMPONENT 3 FROM SKIMMILK

INTRODUCTION TO PART II

The proteose-peptone fraction obtained from bovine milk has been reported to be low in nitrogen and to contain carbohydrate (Thompson and Brunner, 1959). This would support the classification of proteose-peptone as a glycoprotein. However, to date, no individual components of the proteose-peptone fraction have been isolated and their compositions studied. It is possible that within the proteose-peptone group, components vary in the type and amount of carbohydrate.

In the following experiments, chemical analyses were performed on component 3, obtained from heated and unheated skimmilk. The results of such analyses would reveal if component 3 isolated from proteose-peptone is similar to the component 3 isolated from unheated skimmilk. The amino acid composition was determined with the use of Beckman Model 120C Amino Acid Analyzer. Independent analyses were performed on the amino acids tryptophan and cystein. For quantitative determinations of the carbohydrate, colorimetric procedures were adopted, using standard curves constructed from known carbohydrate substances. The carbohydrate moities of component 3 fraction were identified by means of paper chromatography. Physical characterization, i.e., sedimentation-velocity, equilibrium molecular weight, ionic mobility, and diffusion coefficient, was performed on component 3 from heated skimmilk.

EXPERIMENTAL

Apparatus

Amino acid analyses were performed on a Beckman (Spinco) model 120C Amino Acid Analyzer. Spectrophotometric measurements were made with a Beckman DK-2 ratio-recording spectrophotometer. A micro-Kjeldahl distillation apparatus was used for hexosamine determination. The hydrogen ion concentrations of various buffers and solutions were determined with a Beckman Zeromatic pH meter using a glass electrode. Materials were dried in a temperature controlled Cenco vacuum oven.

Sedimentation-velocity and sedimentation-equilibrium studies were performed in a Spinco Model E analytical ultracentrifuge equipped with an RTIC temperature control unit and a phase plate as a schlieren diaphragm. Centrifugation was performed in an An-D dural rotor and a General Electric AH-6 mercury lamp served as the light source. A Starrett microsyringe-burette was used to fill the ultracentrifuge cell for short-column equilibrium experiments.

Chemicals

Unless otherwise specified, all chemicals were reagent grade quality. The chemicals and their suppliers are given as follows: tris (hydroxylmethyl) amino methane from Sigma Company; tryptophan from Nutritional Biochemical Company; p-dimethylaminobenzaldehyde from Eastman Kodak Company;

Ellman's reagent, 5-5' di-thiobis (2-nitrobenzoic acid) from Sigma Company; L-cysteine from Nutritional Biochemical Company; acetylacetone from Fisher Scientific Company; glucosamine-HCl, galactose, fucose from Nutritional Biochemical; thiobarbituric acid from Eastman Kodak Company; synthetic N-acetylneuraminic acid from Carbiochemical Corporation; triphenyltetrazolium chloride from Eastman Kodak Company; sodium barbital (veronal) from Fisher Scientific Company; guanidine HCl from Eastman Kodak Company.

Chemical Methods

Amino Acid Analysis

Component 3 was analyzed for amino acids with a Beckman (Spinco) Model 120C amino acid analyzer. The protein was first hydrolyzed with strong mineral acid. During breakdown of the protein polypeptide chain into amino acids, reactions involving degradation of amino acids, interactions with sugar residues in glycoprotein and rearrangement of bonds might occur. Amino acids differ in their ease of release from polypeptide chain. Thus, amino and imido acids having no side chain groups, i.e., glycine, alanine, valine, leucine, isoleucine, phenylalanine, and proline are rather stable in hot mineral acid solution and require longer time of hydrolysis. On the other hand, the hydroxy amino acids, serine and threonine, are gradually degraded during hydrolysis. Consequently, the choice of the variables time and

temperature is a compromise between sufficiently complete hydrolysis of the protein and minimizing the loss of certain amino acids. Two sets of hydrolytic conditions, i.e., 6 N HCl at 100° C for 20 hours and for 70 hours, were employed in these analyses. In case of a glycoprotein, intermediate sugar degradation products are formed during mineral acid hydrolysis. These are capable of reacting with the amino acid residues, although the rate of reaction is very slow in strong acid solution. This type of interaction can be minimized by using a dilute concentration of the glycoprotein (0.1% in 6 N HCl) in the hydrolysis step.

The amino acid analyses were performed on 20 and 70 hour acid hydrolysate. A protein concentration of about 0.06% in 6 N HCl was used in hydrolysis which were conducted at 110° C for 20 and 70 hours in vacuum sealed tubes. The hydrochloric acid was removed by evaporation at reduced pressure in a warm water bath. The dried amino acid residue was dissolved in dilutor buffer (pH 2.2) and an aliquot applied to the appropriate resin column of the analyzer. Standard chromatograms of known amino acid composition were obtained one to two days prior to the actual runs of the samples, using the same buffer and ninhydrin solutions.

Nitrogen

The digestion mixture consisted of 5 gm. SeO_2 and 5 gm. $CuSO_4 \cdot 5H_2O$ in 500 ml of concentrated H_2SO_4. The protein (5-10 mg) was digested with 3 to 4 ml of the

mixture in a digestion flask. This operation was performed over a gas flame until the mixture was clear. Then, after cooling, one ml of H_2O_2 (30%) was added and the mixture was heated for one hour. The solution was cooled and diluted with 10 ml water. The digestion flask was connected to the distillation apparatus through ball and socket fitting. Twenty-five milliliters of a 40% NaOH solution was added to neutralize the acid. Ammonia was steam distilled into 15 ml of a 4% boric acid solution containing 3 drops of the indicator mixture (400 mg bromcresol green and 40 mg methyl red in 100 ml of 95% ethanol). Standard 0.01974 N HCl was used to titrate the ammonia-borate complex. The normality of the standard HCl solution was determined by titration with standard tris (hydroxymethyl) amino methane. Deionized water was used as a blank. Tryptophan with a known nitrogen content, was used to evaluate the recovery.

Phosphorus

Phosphorus was determined colorimetrically by the method of Sumner (1944). Protein was dried in a vacuum oven over P_2O_5 and weighed (5 go 10 mg) into a test tube. Two and two tenths milliliters of a 50% H_2SO_4 was added. The mixture was digested in sand bath over an electric burner, until SO_3 fumes appeared. It was heated for another 20 minutes. After cooling, 4 drops of 30% H_2O_2 solution was added and heating was continued for 20 minutes. The H_2O_2 treatment was repeated, if necessary, until the

solution was clear. The solution was heated for one hour to remove all H_2O_2. The digestion mixture was transferred quantitatively to a 50 ml volumetric flask. Five ml of a 6.6% ammonium molybdate solution was added. Then four ml of $FeSO_4$ solution [5 gm $FeSO_4(H_2O)_7$ in 50 ml water containing 1 ml 7.5 N H_2SO_4] was added. The final volume was made up to 50 ml with deionized water. Transmission values were read with a Cenco-Sheard-Sanford photelometer equipped with a filter with maximum transmission at 650 mμ. The standard curve was constructed from values obtained with anhydrous potasium phosphate (K_2HPO_4) as a source of phosphorus. The standard was dissolved in 5 ml of 7.5 N H_2SO_4 solution before addition of ammonium molybdate. Deionized water was used as a blank.

Sulfur

Total sulfur was determined by Spang Microanalytical Laboratory, Ann Arbor, Michigan from 10 mg samples by combustion in a Parr bomb.

Tryptophan

Tryptophan is unstable under the usual conditions of acid hydrolysis employed in amino acid determination. Consequently, a separate method of analysis for tryptophan is required. Generally, three methods are available. One involves an alkaline hydrolysis in which tryptophan is relatively stable and subsequent analysis on the amino acid

analyzer (Noltmann, 1962). Also, tryptophan can be determined by a direct spectrophotometric method at wavelengths between 280 and 315 mμ in the ultraviolet region (Beaven and Holiday, 1952). This method has the advantage of avoiding hydrolysis, however, a clear protein solution is required. Third method consists of a colorimetric determination of tryptophan with p-dimethylaminobenzaldehyde (Spies and Chamber, 1949). This method is uncomplicated, sensitive and reproducible, thus it was the method adopted for the tryptophan analysis made here.

Protein (5 to 10 mg) was dissolved in 10 ml of 0.1 N NaOH. Eight millliliters of 24 N H_2SO_4 and 1.0 ml of 2 N H_2SO_4, containing 30 mg of p-dimethylaminobenzaldehyde, were mixed and cooled to 25° C. The sample solution was added to this solution. The solution, after shaking and cooling to 25° C, was kept in the dark at 25° C for one hour. To this solution, was added 0.1 ml of 0.04% sodium nitrite solution. The solution was shaken, and the color was allowed to develop for 30 minutes at room temperature in the dark. Per cent transmission was read at 580 mμ with a DK-2 spectrophotometer. A standard curve was prepared from commercially available tryptophan.

Cysteine

A large number of methods are available for the estimation of the sulfhydryl groups in proteins. This is mainly due to the diversity of reactivity of -SH group with various

chemical reagents. The chemical reagents generally used for -SH group estimation include: 1) mercaptide forming agents, such as p-chloromercuribenzoate (PCMB); 2) oxidizing agents, i.e., performic acid; 3) alkylating agents, such as iodoacetamide, which form a S-carboxymethyl cysteine derivative; and 4) sulfhydryl-disulfide interchanging agent such as Ellman's reagent, 5,5-di-thiobis (2-nitrobenzoic acid) (DTNB). For the quantitative estimation of -SH groups in protein, several methods should be tried to check on their agreements. The agreements between the various methods can be determined by estimating the -SH content of a well characterized protein, such as β-lactoglobulin, which is commercially available in chromatographically homogeneous form. The PCMB-dithizone method (Sasago and others, 1963) and the DTNB method (Ellman, 1959) were tested by the author for estimating -SH group in bovine β-lactoglobulin. Both methods gave approximately 2-SH groups per molecule when determined in the presence of urea. The DTNB method had the advantages of simplicity and high sensitivity, and was the method selected for the detection and estimation of cysteine.

Protein (5 to 10 mg) was dissolved in 1 ml of deionized water. Ellman's reagent, 5-5' di-thiobis (2-nitrobenzoic acid) was prepared by dissolving 39.6 mg of DTNB in 10 ml of phosphate buffer (pH 7.0). A phosphate buffer, pH 8.0 and containing 8 M urea, was also prepared. To one ml of the sample solution, was added 9.0 ml of the phosphate-urea buffer. The solution was shaken and allowed to stand at

room temperature for ten minutes. A three ml aliquot was pipetted into a test tube, to which, was added 0.02 ml of DTNB reagent. The solution was shaken and the per cent transmission was immediately read at 412 mμ, using a DK-2 spectrophotometer. The yellow color formed faded with time. After 24 hours, it was completely colorless. A standard curve was prepared from commercially available L-cysteine.

Hexose

A number of colorimetric procedures are available for the estimation of hexose, which depend on properties other than their reducing behaviors and which do not require prior hydrolysis of the sample. Most of these involve the reaction of sugar with sulfuric acid to give a furfural derivative, yielding colored condensation products with a phenolic base compound. Among these are the orcinol-sulfuric acid method described by Wenzler (1955), the phenol-sulfuric acid method developed by Debois (1956), et al. These methods give optical densities that are proportional to the amount of hexose present over a certain range of hexose concentrations. Other substances present in the sample, such as protein, amino acids, hexosamine and sialic acid, do not interfere with the reaction. Since optical densities vary with the type of hexose, a standard curve should be constructed which contain hexoses in the same proportions as they occur in the sample. We found that the phenol-sulfuric acid method was more sensitive than

the orcinol-sulfuric acid method, in addition to being simple and reproducible. Hence, it was adopted for the hexose analyses of the protein.

Protein was dissolved in deionized water to a concentration of approximately one mg per ml. Two ml aliquot of the sample solution was pipetted into a test tube, to which, was added 0.05 ml of a 80% redistilled phenol solution. Then, 5 ml of concentrated sulfuric acid was added rapidly, the stream of liquid was directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were shaken and allowed to stand at room temperature for 10 minutes. Then, they were placed in a water bath at 25° C for 20 minutes. The per cent transmission of the characteristic yellow orange color was measured at 490 mμ, using a Beckman DK-2 spectrophotometer. Two standard curves were prepared from commercially available galactose and mannose.

Fucose

Fucose, a methylpentose, is a normal constituent of the blood-group substances and is present in many glycoproteins. Fucose has been reported to be present in the proteose-peptone fraction of bovine milk (Thompson and Brunner, 1959). A colorimetric method for fucose determination involves heating the sample with sulfuric acid, followed by addition of cysteine hydrochloride to give a green-yellow color (Wenzler, 1955). The optical densities are read at two wavelengths and

the difference between these values is compared with that given by standard fucose solution. Hexose, hexosamine and pentose do not interfere with the reaction.

Protein (5 to 10 mg) was dissolved in 10 ml of deionized water. A fucose standard solution, containing approximately 20 μg per ml, was prepared. A sulfuric acid-water mixture was made up of six volumes of concentrated sulfuric acid and one volume of water. One milliliter aliquots of the sample solutions were pipetted into test tubes. To these tubes (and to one ml of water for blank and one ml of the fucose standard), were added 4.5 ml of ice cold H_2SO_4 - H_2O solution. The solutions were mixed while maintained in an ice bath. The solutions were heated for exactly three minutes in a boiling water bath and cooled in tap water. Cysteine-hydrochloride solution (0.1 ml of a 3% w/v solution) was added and mixed immediately. The cysteine reagent was omitted from one of the samples to correct for non-specific color development. The solutions were allowed to stand at room temperature for 60 minutes and the per cent transmission was read at 396 and 430 mμ, with a Beckman DK-2 spectrophotometer.

Hexosamine

The analysis of hexosamine in glycoprotein involves, firstly, the hydrolysis of hexosamine from the glycoprotein, followed by the determination of hexosamine in the hydrolysate. The usual hydrolytic conditions employed in the

hexose analysis (1 to 2 N HCl at 100° C for 4 to 5 hours) might result in incomplete liberation of hexosamine. The hydrolytic condition employed in the amino acid analysis (6 N HCl at 110° C for 20 hours) would result in appreciable destruction of hexosamine. The hydrolytic condition usually employed for hexosamine determination, i.e., 4 N HCl at 100° C for 4 to 6 hours, represents a compromise between maximum release of the amino sugars with minimum destruction. A common procedure for the determination of hexosamine is the method developed by Elson and Morgan (1933), whereby acetylacetone is allowed to react with the amino sugar in a hot, mildly alkaline solution. A mixture of pyrroles is obtained, giving a pink color with Ehrich reagent (p-dimethylaminobenzaldehyde). Several modifications of the Elson and Morgan method have been reported (Rondle and Morgan, 1955; Kraan and Muir, 1957; Exley, 1957). One of these, the Cessi method (Cessi and Piliegó, 1960) involves the steam distillation into Ehrlich's reagent of 2-methylpyrrole, a volatile compound that is produced among the mixture of pyrroles. The Cessi method was reported to be highly reproducible (Johansen et. al., 1960) and was the method employed for the hexosamine analysis.

The acetylacetone reagent was prepared by dissolving one ml of colorless, redistilled acetylacetone (boiling point, 138 to 140° C) in 100 ml of 0.5 N sodium carbonate-sodium bicarbonate buffer, containing 0.1 N

sodium chloride. The pH of the buffer should be close to 9.8. Ehrich reagent was prepared by dissolving 80 mg of p-dimethylaminobenzaldehyde in 100 ml of absolute ethanol containing 3.5 ml of concentrated hydrochloric acid. Hydrolysis proceeded as follows: protein (5 to 10 mg) was dissolved in 5 ml of 4 N HCl and hydrolyzed at 100° C for 6 hours under an atmosphere of nitrogen, the hydrolysate was dried in vacuum over NaOH in a dessicator maintained at room temperature. The dried hydrolysate was dissolved in 10 ml of water, a two-milliliter aliquot was pipetted into a small micro-Kjeldahl distillation flask, to which, was added 5.5 ml of acetylacetone reagent (pH 9.8). The solution was heated in a boiling water bath for 20 minutes. After cooling in tap water, the digestion flask was connected to a steam distillation apparatus. Heating was done with a microburner. Portions (2 ml) were distilled into 10 ml volumetric flasks containing 8 ml of the Ehrich reagent. Per cent transmissions were determined 30 minutes later at 545 mμ, with Beckman DK-2 spectrophotometer. A standard curve was prepared from commercially available glucosamine-HCl, which was treated with the acetylacetone reagent as described above.

Sialic Acid

Sialic acids comprise the various N-acetylated and N-acylated-O-acetylated neuraminic acids widely distributed in animals in predominantly bound forms. Among the carbo-

hydrate moities of glycoprotein, sialic acid is the most labile to acid hydrolysis. This is related to the structure of sialic acid which resembles a deoxy-sugar, the glycosides of which have been reported to be hydrolyzed much more readily than other glucose derivatives (Overend et. al., 1962). The sialic acid appears to occupy a non-reducing terminal position in the heterosaccharides chain and is linked ketosically either to hexose or hexosamine (Kuhn, 1960). In the determination of total sialic acid, the usual procedure is to perform the hydrolysis first to release sialic acid from its bound form. The condition employed is a mild acid treatment in 0.1 N H_2SO_4 at 80° C for 30 minutes. The free sialic acid can be determined by several colorimetric procedures, among which the thiobarbituric acid method (Warren, 1959) is the most sensitive and reproducible. The method is based on the periodate oxidation of sialic acid to form cleavage products which react with thiobarbituric acid to give a color compound with an absorption maximum at 549 mμ.

Protein (5 to 10 mg) was dissolved in 10 ml of 0.1 N H_2SO_4 solution and hydrolyzed at 80° C in a water bath for 30 minutes. A four-tenths ml aliquot was pipetted into a test tube, to which was added 0.1 ml of sodium periodate solution (0.2 M sodium meta-periodate in 9 M phosphoric acid). The tubes were shaken and allowed to stand at room temperature for 20 minutes. One milliliter of sodium arsenite solution (10% sodium arsenite in a solution of

0.5 M sodium sulfate-0.1 N H_2SO_4) was added and the tubes shaken until the yellow-brown color disappeared. Three milliliters of thiobarbituric acid solution (0.6% in 0.5 M socium sulfate) was added. The tubes were shaken, capped with marbles, and heated in a vigorously boiling water bath for 15 minutes. The tubes were removed and placed in cold water for 5 minutes, followed by the addition of 4.3 ml of cyclohexanone, which was used for the extraction of the chromophore. The tubes were shaken and the contents were transferred to conically shaped tubes and centrifuged for 3 minutes in a clinical centrifuge. The clear, upper cyclohexanone phase was red and more intense than in the aqueous phase. Per cent transmission of the organic phase was measured at 549 mμ with a Beckman DK-2 spectrophotometer. A standard concentration curve was prepared from commercially available, synthetic N-acetylneuraminic acid.

Paper Chromatographic Identification of Carbohydrate

The colorimetric methods for hexose and hexosamine do not differentiate between the types of hexose and hexosamine in glycoproteins. Hence, a qualitative analysis of the carbohydrate moities was required. Several methods are available for the identification of sugars in biological substances. These include paper chromatography, thin-layer chromatography, gas chromatography of sugar derivatives, and others. The carbohydrate was hydrolyzed from the sample with 1-2 N HCl. The acid and other charged groups, such as

amino acids or peptides that may be present in the hydrolysate, should be removed before the sample is applied on the chromatographic paper. This operation can be accomplished in several ways, such as ion exchange chromatography, repeated lyophilization (in case of HCl), extraction with pyridine or a combination. Paper chromatography was adopted for the tentative identification of hexose and hexosamine in component 3. A mixture of known sugars was chromatographed as a standard.

Purified component 3 (approximately 50 mg) was dissolved in 5 ml of 2 N HCl and hydrolyzed in a sealed tube at 100° C for 6 hours. The hydrolysate was dried by evaporation under reduced pressure in a warm water bath. The dried hydrolysate was dissolved in 5 ml of water and passed through a Dowex 50 8X (H^+ form) column (2.2 x 30 cm) coupled with an Amberite 4B (OH^- form) column (2.2 x 30 cm). The effluent (300 ml) was evaporated to dryness under reduced pressure. The dried material was extracted with 5 ml of pyridine (redistilled) on a steam bath for 5 minutes. Pyridine was removed by evaporation under reduced pressure at temperature below 40° C. The extracted sugar residue was dissolved in a minimum amount of 10% propanol, and applied to the chromatographic paper (Whatman no. 1). A standard mixture of known sugars was spotted alongside the sample. The relative positions of the known sugars in the mixture were ascertained by spottings of individual sugars in the chromatogram. The descending chromatogram was run for 18 hours, at room temperature, using a solvent mixture made up of butanol-pyridine-water (6:4:3, v/v). The chromatogram was dried at 100° C

for 10 minutes and developed at 60° C in a moist atmosphere with 2% triphenyltetrazolium chloride in an equal volume of 1 N NaOH.

Physical Methods

Free-Boundary Electrophoresis

This technique is used to determine the ionic mobility of a purified protein preparation and to estimate its isoelectric pH value. Electrophoretic mobilities are calculated from measurements made from the position of initial boundary on the descending and ascending patterns, as follows:

$$\mu = \frac{dAk}{tIRm}$$

where μ is the electrophoretic mobility in cm^2 $volt^{-1}$ sec^{-1}, d the distance measured from the initial boundary in cm, A the cross-sectional area of the electrophoretic cell in cm^2, k the conductivity cell constant, t the time in seconds, I the current in amperes, R the resistance of the buffer in ohms, and m the magnification of the optical system. The protein solution should be clear in order to obtain a good photographic pattern. Also, the protein solution should be dialyzed against the buffer until ionic equilibrium is attained. For estimation of isoelectric point of protein, a series of electrophoretic runs with buffer pH ranging from basic to acidic (so that the net charge on protein changes from negative to positive, respectively) are

employed. The isoelectric point of the protein is estimated from a plot of ionic mobility against pH. The preparations of the buffers used in free-boundary electrophoretic runs are shown in the Appendix, Part II.

Ultracentrifugation

Sedimentation-Velocity: The sedimentation-velocity method is used for the determination of the sedimentation coefficient of the molecule and for providing information about the purity of the material under investigation. The sedimentation coefficient is defined as the velocity of the sedimenting molecule per unit centrifugal field. The ultracentrifuge is operated at top speed, and the movement of the boundary across the cell is recorded in the photographs which are taken at periodic intervals. Sedimentation coefficient is calculated from the following equation,

$$s = \frac{2.303}{w^2} \log \frac{x}{t}$$

where s is the sedimentation coefficient in sec, x the distance of the boundary in cm from the axis of rotation, t the time in secs and w the angular velocity in radians per sec. Generally, the sedimentation coefficient is expressed in units of 1×10^{-13} sec and the unit 1×10^{-13} sec has been termed 1S, where S is the Svedberg. Experimentally, a plot of log x against t gives essentially a straight line, the slope of which is used to calculate the sedimentation coefficient. For many proteins, the sedimentation coefficient

is dependent on concentration (Schachman, 1959). Determinations are made at different concentrations and the sedimentation coefficient is estimated at infinite dilution.

Sedimentation-Equilibrium: Sedimentation-equilibrium is attained when the material migrating across a given surface in a centrifugal direction is balanced by the transport centripetally due to diffusion. Sedimentation-equilibrium is used for calculating the molecular weight of a protein. The classical sedimentation-diffusion equilibrium requires a long running period for attainment of equilibrium (VanHolde and Baldwin, 1958). Archibald (1947) has shown that molecular weights can be calculated from data obtained during the early stages of a centrifugal run. He pointed out that the solute does not leave the centrifuge cell either at the meniscus or the bottom of the cell and therefore the conditions for equilibrium are fulfilled at these two locations in the cells at all times of the run. The Archibald procedure, generally known as the approach-to-equilibrium method, has been frequently applied for molecular weight study (Erlander and Foster, 1959). The method requires knowledge of the concentration of solute at the bottom and meniscus of the cell. It should be noted that determination of molecular weight by this method should be made over a range of protein concentrations, since calculation based on one concentration may be unsound unless the protein solution is truly monodispersed. In the case of a polydisperse system,

the apparent weight-average molecular weight is dependent on concentration. The apparent weight-average molecular weight is plotted against concentration and the $M^{o}_{w,app}$ at infinite dilution is calculated by extrapolation to zero concentration. Where the apparent weight-average molecular weight shows dependence on protein concentration, attempts are made to determine molecular weight in the presence of a dissociating agent, such as 5 M guanidine-HCl, in the hope of obtaining the molecular weight of the light component that is nearly independent or shows a less degree of dependence on concentration.

Sedimentation-velocity and sedimentation-equilibrium studies were performed in a Spinco Model E, analytical untracentrifuge equipped with an RTIC temperature-control unit and a phase plate as a schlieren diaphragm. A synthetic-boundary cell was used in all sedimentation-velocity runs. Equilibrium studies were performed in the double-sector cell. Centerpieces were of the filled-Epon type. A false bottom of FC-43 fluorocarbon oil was employed in the short-column equilibrium experiments. The sedimentation-velocity studies were performed at 20° C with rotor speeds of 59,780 r.p.m. The photographic plates were read with a Nikon microcomparator capable of measuring to less than 0.002 mm. A sample calculation of the equilibrium molecular weight by the sedimentation equilibrium method is shown in the Appendix, Part II.

Diffusion Coefficient: The diffusion coefficient is the proportionality factor relating the rate of transfer of material across unit cross section to the rate of concentration with respect to the distance (the concentration gradient) at the given cross-section. The diffusion coefficient of a protein is a physical parameter relating to the size and shape of the molecule. The diffusion coefficient can be obtained employing the schlieren optics in Tiselius free-boundary electrophoretic cell or the Beckman Model E ultracentrifuge. In the schlieren optics, concentration gradient is proportional to the peak height and area. Diffusion coefficient can be calculated from measurements of peak height and area at various time intervals, according to the following formula,

$$D = \frac{m^2 A^2}{4\pi t H^2}$$

where D is the diffusion coefficient in cm^2/sec, A is the area in cm^2, H is the peak height in cm, t the elapsed time in sec and m, the magnification of the optics. With Tiselius free-boundary electrophoretic cell, the area is measured by weighing the enlarged photographic patterns. With Beckman Model E ultracentrifuge, the area is measured from the photographic plates, using a Nikon microcomparator. The quantity $m^2A^2/4\pi H^2$ is plotted against time. The diffusion coefficient is obtained from the slope of the line. The diffusion coefficient of a protein often depends on protein concentration (Greenberg, 1951). Hence, diffusion

runs are made at various protein concentrations. In the following diffusion runs, a Tiselius cell was used for the protein in veronal buffer, pH 8.6, ion strength = 0.1 and a Beckman Model E ultrancentrifuge was used for the protein in veronal-5 M guanidine hydrochloride, since large quantity of guanidine hydrochloride was not available at the time for runs in the Tiselius cell.

RESULTS AND DISCUSSIONS

Chemical Composition

The amino acid compositions of component 3 from heated and unheated skimmilk are shown in Table 3. The analyses were reported as g residue/100 g protein and as number of residues/1000 residues. The weight percentages of the amino acid residues were based on a nitrogen content of 13.1% and 13.2% for component 3 from heated and unheated skimmilk, respectively, as determined by micro-Kjeldahl nitrogen determination. The second method of expression, i.e., number of amino acid residues/1000 total residues, was used for comparing the similarity of the protein isolated from heated and unheated skimmilk. This method of presentation relates the proportions of amino acid residues with respect to one another, hence, is not affected by experimental errors such as weighing or losses during transfers.

A summation of the residue weights of the amino acids, together with the values for hexose, hexosamine, sialic acid and phosphorus, accounted for approximately 95% of the protein. The protein portion constituted about 80% by weight of the glycoprotein, the remaining portion was essentially carbohydrate. Independent analyses on the amino acids tryptophan and cysteine indicated that component 3 contained negligible or trace amounts of tryptophan and cysteine. Analytical data obtained with the Beckman Model 120C amino acid analyzer revealed that component 3 was low in the aromatic acids

tyrosine and phenylalanine; low in the sulfur-containing amino acids (no cystine and low methionine); and high in glutamic acid, lysine and leucine. Two small unidentified peaks (2% by weight of total) appeared in the chromatograms from both basic and neutral columns. The chromatogram of the 20 hour hydrolysate showed qualitative evidence of glucosamine and galactosamine, the glucosamine being in higher concentration than the galactosamine.

The amino acid compositions, in number of residues per 1000 total residues, of component 3 from heated and unheated skimmilk, are presented in the form of the amino acid profiles as shown in Figure 14. These profiles matched surprisingly close to one another, hence their amino acid compositions were very similar. This rendered strong evidence that component 3 was present as a native glycoprotein in milk and that its amino acid composition was essentially unaltered by the heat treatment employed in its preparation.

Elementary analyses of component 3 from heated and unheated skimmilk are shown in Table 4. Again, their nitrogen and phosphorus contents were similar. The nitrogen content of 13.1% for component 3 was low compared with the nitrogen value reported for proteose-peptone, i.e., (13.7 to 13.9%) by Thompson and Brunner (1961). The phosphorus content of component 3, 0.5% was also low compared with their value of 1.1% P. Elementary analyses of the other proteose-peptone components, i.e., component 5 and 8 (Kolar,

Ph. D. Thesis, 1967), indicated that these components contained higher concentrations of P than component 3. A sulfur analysis, performed by the Spang Analytical Laboratories, identified 0.59% S for component 3. This value is higher than can be accounted for from methionine sulfur.

The amino acid composition of component 3 indicated that it was an acidic protein, i.e., the sum of glutamic and aspartic acid residues was greater than the sum of lysine, histidine and arginine residues. The low isoelectric point, i.e., pH 3.7, estimated from free-boundary electrophoresis studies supported this conclusion. Comparing the chemical composition of component 3 with other minor protein fractions, (i.e., Whitney, 1958; Thompson and Brunner, 1962), component 3 had a lower nitrogen content and contained a sizeable amount of carbohydrate, while most of the minor protein fractions contained much lower concentrations of carbohydrate. The amino acid composition of component 3 was characterized by its low aromatic amino acid content compared with other known milk proteins. Recently, a phosphoglycoprotein from bovine milk whose chemical composition and ultracentrifugal properties corresponded to the major component of the proteose-peptone fraction was reported (Bezkorovainy, 1965). Its amino acid composition showed high glutamic acid, aspartic acid and isoleucine contents. It contained 9.5% carbohydrate and had a sedimentation constant of 0.8 at pH 7.0 at 1% concentration. Although the amino acid composition, carbohydrate

content, and ultracentrifugal properties of the phosphoglycoprotein differ from that of component 3 prepared for this study, it is conceivable that other proteose-peptone components, in their predominant forms, or in varying associations with one another, might give a molecular species whose chemical and physical properties were similar to the phosphoglycoprotein reported. An acrylamide gel electrophoretic run of this phosphoglycoprotein, and compared with various proteose-peptone components, would reveal if similarities exist among these fractions.

The carbohydrate contents of component 3 from heated and unheated skimmilk are shown in Table 5. The standard curves for the carbohydrate analyses are shown in the Appendix, Part II. Component 3 from unheated skimmilk possessed a higher hexosamine and lower hexose content than component 3 from heated skimmilk. Possibly, a slight degree of sugar degradation, such as deamination of the hexosamine, occurred as a result of the heat treatment employed. The sugar content of 17.3% in component 3 was among the highest reported for milk glycoproteins. The caseino-glycopeptide released from k-casein by treatment with rennet, had a sugar content of 28.1% (Alais and Jolle's, 1961). k-Casein was reported to contain 5.0% carbohydrate (Alais and Jolle's, 1961). Conceivably, the action of some proteolytic enzymes, possibly rennin, pepsin and/or others, might release a glycopeptide from component 3 with a considerably higher sugar content. The proteose-peptone fraction was reported to

contain 6.8% carbohydrate (Thompson and Brunner, 1959). A comparison of the sugar contents of component 3 with those of the other proteose-peptone components (Kolar, Ph. D. Thesis, 1967) indicated that component 3 had the highest carbohydrate content among the proteose-peptone components. Paper chromatographic identification of the sugar moities in component 3 is shown in Figure 15. The chromatograms were interpreted to indicate the presence of galactosamine, glucosamine, galactose, mannose and fucose as the major carbohydrates in component 3.

Minimum molecular weights of component 3 from heated skimmilk were calculated from chemical analyses of known constituents, as shown in Table 6. An independent determination of molecular weight by ultracentrifugal methods permitted a calculation of the number of residues of each constituent per mole of component 3. Assuming accurate chemical analyses and a reliable molecular weight determination, the number of residues per mole of protein should approach an integral value. The minimum molecular weights calculated from determinations of sialic acid (as N-acetylneuraminic acid), hexosamine, phosphorus, sulfur and tyrosine (the limiting amino acid in component 3) indicated that the above constituents existed in roughly integral ratios of 3:1:2:2:7 in that order.

Physical Properties of Component 3

Electrophoretic Characteristics

The ionic mobilities of component 3 from heated skim-milk, calculated from the free-boundary electrophoretic patterns, in buffers ranging from pH 8.6 to 2.0, ion strength = 0.2, are shown in Table 7. The free-boundary patterns are shown in Figure 16. The ionic mobility in veronal buffer, pH 8.6 and ion strength = 0.2, is -3.5 cm^2 sec^{-1} $volt^{-1}$ x 10^5 from the descending channel. Thompson and Brunner (1961) reported the mobility of component 3 from the electrophoretic patterns of proteose-peptone, in veronal buffer, pH 8.6 and ion strength = 0.1, to be -2.0 Tiselius units (from descending channel), while Larson (1955), using similar buffer system, reported a value of -3.0 Tiselius units (from descending channel). It is possible that ionic mobility of a protein component would vary slightly depending on whether it is present singly or in association with other protein components. Also, equilibrium dialysis is important in ionic mobility determination. In all the electrophoretic runs in buffers ranging from pH 8.6 to 2.0, component 3 appeared as a single peak. A plot of ionic mobilities (values from descending patterns) against pH is shown in Figure 17. The mobility decreases to a minimum as the pH of the buffer approaches the isoelectric point of the protein. The isoelectric point, obtained from the intercept of the curve on the pH-axis, was estimated at

pH 3.7. The low isoelectric point of component 3 would fit its description as an acidic glycoprotein. This acidic character agrees with its chemical constitution, which shows an excess of the acidic residues over the basic residues.

Ultracentrifugal Characteristics

Diffusion Coefficient: The diffusion coefficients of component 3 from heated skimmilk in veronal and veronal-5 M guanidine hydrochloride buffers, at various protein concentrations, are shown in Table 8. The diffusion coefficients were corrected to water at 20° C. A plot of diffusion coefficients against protein concentrations is shown in Figure 18. The diffusion coefficients of component 3 increased with increases in protein concentrations. Possibly, molecular aggregation decreased with an increase in protein concentration, as would be evident in subsequent sedimentation-equilibrium studies of molecular weights. The smaller molecular aggregate obtained at increased protein concentration would tend to give a higher diffusion coefficient. A five-to-eight-fold increase in the diffusion coefficient of component 3 was obtained in veronal buffer containing 5 M guanidine hydrochloride, compared with straight veronal buffer. Conceivably, in veronal buffer containing the dissociating agent, a low molecular-weight species or monomer unit of component 3 existed. The light component would tend to give a higher diffusion coefficient than a large molecular polymer. The diffusion coefficient of

component 3, in veronal buffer, at infinite dilution, was estimated at $D_{20,w} = 1.80 \times 10^{-7}$ cm^2/sec, and in veronal-5 M guanidine hydrochloride, at infinite dilution, was estimated at $D_{20,w} = 11.5 \times 10^{-7}$ cm^2/sec.

Sedimentation Coefficient: The sedimentation coefficients of component 3 from heated skimmilk in veronal and veronal-5 M guanidine hydrochloride, at various protein concentrations, are shown in Table 9. A plot of the sedimentation coefficients against protein concentrations is shown in Figure 19. The sedimentation coefficient decreased with increases in protein concentrations. The sedimentation coefficient was considerably reduced in the presence of the dissociating agent 5 M guanidine hydrochloride, indicating that the polymer-monomer equilibrium was shifted toward the low molecular-weight species in a dissociating system. A single boundary appeared in the sedimentation patterns of component 3 at protein concentrations greater than 5 mg/ml. At dilute concentrations of less than 5 mg/ml, high molecular-weight contaminants, possibly some denatured euglobulin or polymorphic species of component 3, appeared as spikes (negligible areas), in the sedimentation patterns. The sedimentation coefficient, at infinite dilution, in veronal buffer, was estimated at $S_{20,w} = 4.0$ Svedberg, and that at infinite dilution, in veronal-5 M guanidine hydrochloride, was estimated at $S_{20,w} = 1.62$ Svedberg.

Sedimentation-Equilibrium Studies of Molecular Weight

The equilibrium molecular weight of component 3 from heated skimmilk, in veronal buffer, is dependent on protein concentration as shown in Table 10. A plot of equilibrium molecular weight against protein concentration is shown in Figure 20 and 21. It is interesting to note that the molecular weight decreased with increases in protein concentrations. k-Casein, a glycoprotein, also exhibits similar behavior (Swaisgood, 1964). The equilibrium molecular weight of component 3, at infinite dilution, in veronal buffer, was estimated at 200,000. This value approximates the calculated molecule weight of 207,000 obtained from sedimentation and diffusion coefficients of component 3, at infinite dilution, in the same veronal buffer (Svedberg equation).

Presumably, the high molecular weight of component 3 in veronal buffer is due to polymer formation. To study the molecular weight of the smaller unit or light component, a dissociating agent, 5 M guanidine hydrochloride in veronal buffer, was employed for the purpose of shifting the polymer-monomer equilibirum toward the lower molecular-weight species. The equilibrium molecular weight of component 3 in veronal-5 M guanidine hydrochloride at various protein concentrations is shown in Table 10. The equilibrium molecular weight of component 3 was considerably reduced in the presence of the dissociating agent, indicating that

disaggregation of the polymeric species of component 3 occurred. A plot of equilibrium molecular weight of component 3 against protein concentration in veronal-5 M guanidine hydrochloride is shown in Figure 21. In the dissociating system employed, the equilibrium molecular weight again exhibited dependence on protein concentration, although the degree of dependence of molecular weight on protein concentration was less in the presence of the dissociating agent. The protein was not monodispersed in veronal-5 M guanidine hydrochloride buffer as evidenced by an M_z/M_w ratio of greater than one, the M_z/M_w ratio being a measure of the degree of polydispersity (Tanford, 1961). Possibly, other dissociating systems, such as 67% acetic acid-0.15 M NaCl, anhydrous formic acid, and others, should be employed, which might further reduce the dependence of molecular weight on protein concentration, and thus permit a determination of the molecular weight of the monodispersed protein.

Alternately, the molecular weight of the light component or small unit of component 3 might be obtained according to Trautman's treatment of approach-to-equilibrium method (Swaisgood, Ph. D. Thesis, 1963). Here, the sedimentation equilibrium runs are conducted at various rotor speeds and at various protein concentrations. The molecular weight of the small component is calculated from the slope of the plot at rotor speeds where only the small component leaves the meniscus.

The equilibrium molecular weight of component 3, at infinite dilution, in veronal 5 M guanidine hydrochloride is estimated at 40,000 (Figure 21). A minimum molecular weight calculated from the tyrosine determination (the limiting amino acid in component 3) gave a value of approximately 22,000. Since 40,000 does not represent the molecular weight of the monodispersed protein, it is likely that the molecular weight of the small component or the monodispersed protein might lie in the range of 22,000 to 40,000.

SUMMARY TO PART II

Component 3 is a whey glycoprotein. Its amino acid composition was characterized by a relatively high content of glutamic acid, leucine and lysine and a low content of aromatic and sulfur-containing amino acids. Its carbohydrate content of 17.3% was high compared with other milk glycoproteins. The carbohydrate moieties consisted of galactosamine, glucosamine, galactose, mannose, fucose and sialic acid.

Component 3 had an isoelectric point of 3.7, which classified it as an acidic glycoprotein. The ionic mobility in veronal buffer, pH 8.6 and $\Gamma/2 = 0.2$, was 3.5 Tiselius unit. The sedimentation coefficient was $S_{20,w} = 4.0$, at infinite dilution in veronal buffer, pH 8.6 and $\Gamma/2 = 0.1$. The diffusion coefficient was $D_{20,w} = 1.8$, at infinite dilution, in veronal buffer, pH 8.6 and $\Gamma/2 = 0.1$. Sedimentation-equilibrium studies indicated that the molecular weight was concentration dependent. The equilibrium molecular weight was estimated at 200,000, at infinite dilution, in veronal buffer, pH 8.6 and $\Gamma/2 = 0.1$. This agreed well with a molecular weight of 207,000 obtained from sedimentation-velocity and diffusion runs. In the presence of a dissociating agent, the polymer-monomer equilibrium was shifted toward the low molecular-weight component. The equilibrium molecular weight was estimated at 40,000 in veronal-guanidine hydrochloride. However, even

in such dissociating agent, the molecular weight still showed concentration dependence, indicating that the protein was not completely monodispersed. A minimum molecular weight estimated from the limiting amino acid gave a value of 22,000. The molecular weight of the monodispersed protein was estimated between 22,000 and 40,000.

TABLE 3

Amino acid composition of component 3
(heated and unheated preparations)

Residue	Grams Residue/ 100 g Protein[a]		Number Residues/ 1,000 residues[b]	
	Heated	Unheated	Heated	Unheated
Lysine	7.31	7.55	84.65	87.04
Histidine	3.27	3.19	35.33	34.44
Arginine	4.04	4.02	38.42	38.14
Unidentified Peak	1.79	1.85	18.70	19.15
Aspartic Acid	6.47	6.42	83.32	82.50
Threonine[c]	5.58	5.40	78.02	74.93
Serine[c]	6.26	6.03	96.28	91.39
Glutamic Acid	14.40	14.22	165.91	161.98
Proline	4.79	4.82	73.31	73.57
Glycine	1.07	1.15	27.82	29.60
Alanine	2.16	2.16	44.16	45.11
Unidentified Peak	0.27	0.27	4.12	4.16
Valine	2.16	2.48	32.39	37.01
Methionine	1.60	1.29	18.11	14.53
Isoleucine	4.82	4.99	63.30	65.17
Leucine	8.15	8.50	106.87	111.26
Tyrosine	0.74	0.74	6.18	6.36
Phenylalanine	2.28	2.29	23.00	23.09
Tryptophan	Trace	Trace	--	--
Cysteine	Trace	Trace	--	--
W_{aa} =	77.06	77.37		
			N_i = 1000.00	999.3
Total Carbohydrate (weight %)	17.30	17.20		
P (as H_2PO_3)	1.30	1.31		
ΣW =	95.66	95.87		

[a]Weight percentage of the i^{th} amino acid residue, based on a nitrogen content of 13.1% and 13.2% for component 3 from heated and unheated preparations respectively, as determined by micro-Kjeldahl nitrogen determination.

[b]Obtained by summation of the moles of the i^{th} amino acid from the chromatogram, corrected to 1000 total amino acid residues.

[c]Values extrapolated to zero concentration.

TABLE 4

Elementary analysis of component 3 from heated and unheated skimmilk

Element	Heated preparation (%)	Unheated preparation (%)
N	13.1	13.2
P	0.5	0.5
S	0.59	--

TABLE 5

The carbohydrate contents of component 3 from heated and unheated skimmilk

Carbohydrate	Heated preparation (%)	Unheated preparation (%)	Method
Hexose	7.2	6.5	Phenol-sulfuric
Hexosamine	6.0	6.6	Cessi
Sialic acid	3.0	2.9	Warren
Fucose	1.1	1.2	Dische
Σw_1	17.3	17.2	

TABLE 6

Minimum molecular weights of component 3 (heated preparation) estimated from chemical analysis

Component	Weight percentage	Minimum molecular weight
Sialic acid	3.0	10,300
Hexosamine	6.0	2,990
Phosphorus	0.50	6,200
Sulfur	0.59	5,450
Tyrosine	0.75	21,800

TABLE 7

Electrophoretic properties of component 3
(heated preparation) in various buffers

Buffer system ($\Gamma/2 = 0.2$)	pH	Protein concentration (%)	Electrophoretic mobility[a] (1×10^{-5} cm^2 sec^{-1} v^{-1})		
			Descending	Ascending	Average
Veronal	8.5	1.5	-3.48	-4.17	-3.83
Phosphate	7.0	1.5	-1.87	-2.91	-2.39
Phosphate	6.0	1.5	-1.67	-2.35	-2.01
Acetate	5.0	1.5	-1.56	-1.75	-1.66
Acetate	4.0	1.5	-0.38	-0.47	-0.43
Glycine:HCl	3.5	1.5	+0.42	+0.78	+0.60
Glycine:HCl	3.0	1.5	+1.17	+2.15	+1.66
Glycine:HCl	2.5	1.5	+1.54	+2.61	+2.08
Glycine:HCl	2.0	1.5	+1.80	+3.12	+2.46

[a]Run at temperature of 0 to 2° C.

TABLE 8

Diffusion coefficient of component 3 (heated preparation) in veronal and veronal-5 M GU buffers

Buffer	Protein concentration (mg/ml)	$D_{20,w} \times 10^7$
Veronal (pH 8.6, $\Gamma/2 = 0.1$)	4.5	3.15[a]
	5.5	3.47[a]
	6.0	3.76[a]
Veronal-5 M GU	6.4	18.7[b]
	7.5	20.1[b]
	8.9	22.0[b]
	10.1	22.6[b]

[a]Obtained from ascending pattern of the Tiselius cell, run at 3° C, corrected to water at 20° C.

[b]Obtained from Beckman Spinco Model E ultracentrifuge, run at 20° C, corrected to water at 20° C.

TABLE 9

Sedimentation coefficient of component 3 (heated preparation) from veronal and veronal-5 M GU buffers

Buffer	Protein concentration (mg/ml)	$s_{20,w} \times 10^{13}$
Veronal (pH 8.6, $\Gamma/2 = 0.1$)	3.1	3.61
	4.4	3.44
	5.1	3.37
	6.1	3.20
Veronal-5 M GU	5.0	1.47
	7.5	1.39
	8.9	1.33
	10.1	1.32

TABLE 10

Equilibrium molecular weight data for component 3 (heated preparation) in veronal and veronal-5 M GU buffers

Buffer	Protein concentration (mg/ml)	$M_w \times 10^{-4}$	M_z/M_w
Veronal (pH 8.6, $\Gamma/2 = 0.1$)	3.1	12.30	--
	4.4	9.38	--
	5.1	7.53	--
	6.1	5.39	--
Veronal-5 M GU	5.0	3.15	1.49
	6.4	2.90	1.20
	7.5	2.76	1.56
	8.9	2.51	1.57
	10.1	2.28	2.07

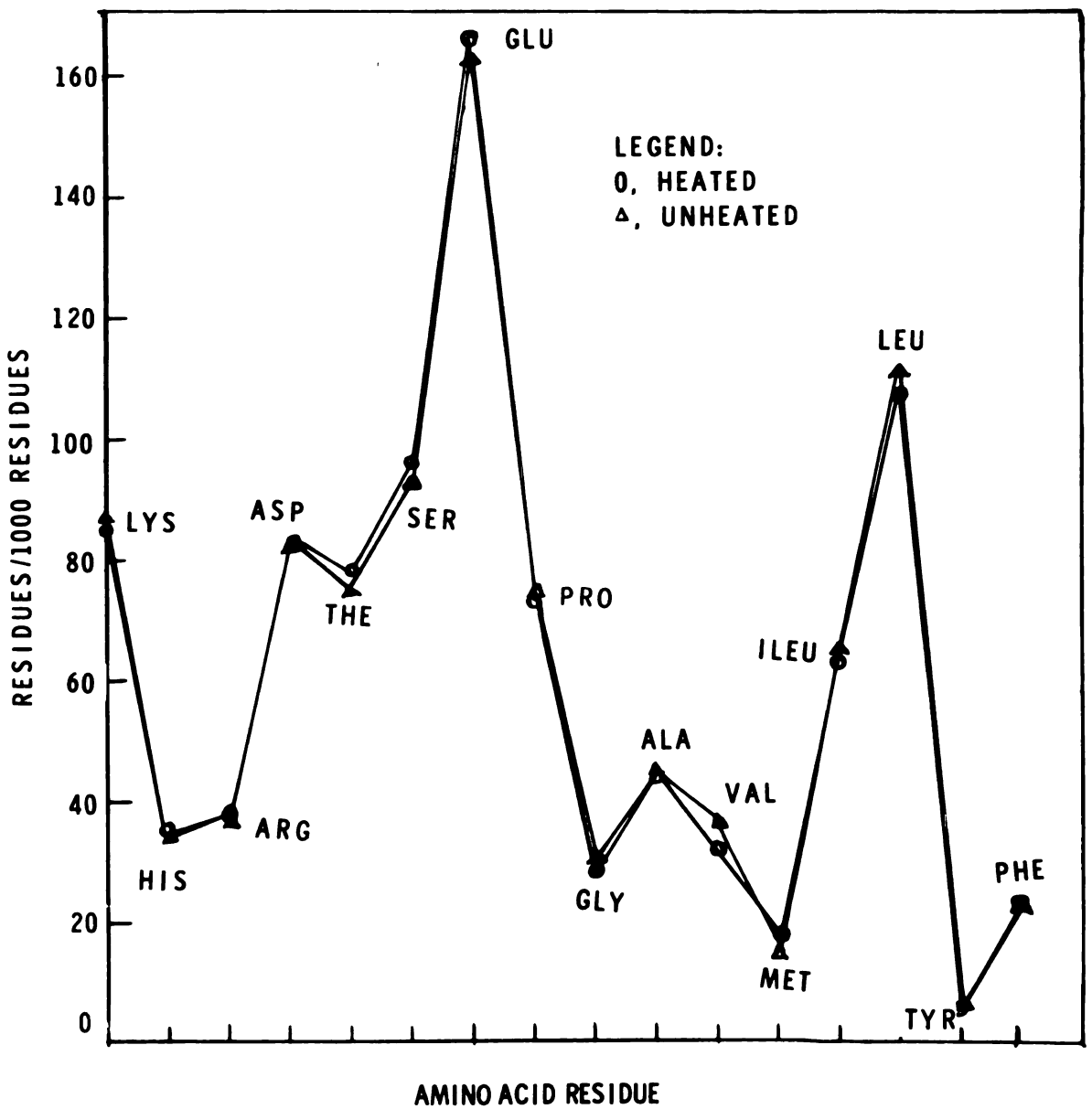
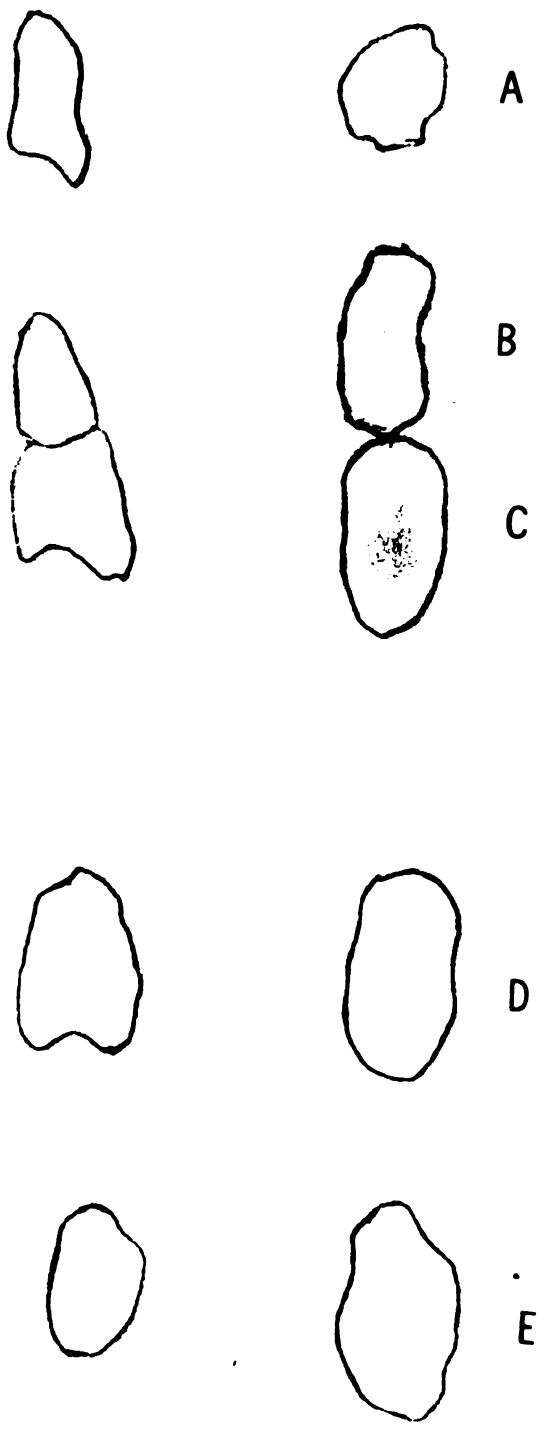


Figure 14. Amino acid profiles of component 3 from heated and unheated skimmilk.

Figure 15. Paper chromatogram of the hydrolysis-released carbohydrate moities of component 3 (heated preparation). Descending pattern; Whatman no. 1 chromatography paper; solvent system: butanol-pyridine-water (6:4:3 v/v); stained with triphenyltetrazolium chloride. Legend: A, galactosamine; B, glucosamine C, galactose; D, mannose; E, fucose.

SAMPLE	STANDARD MIXTURE



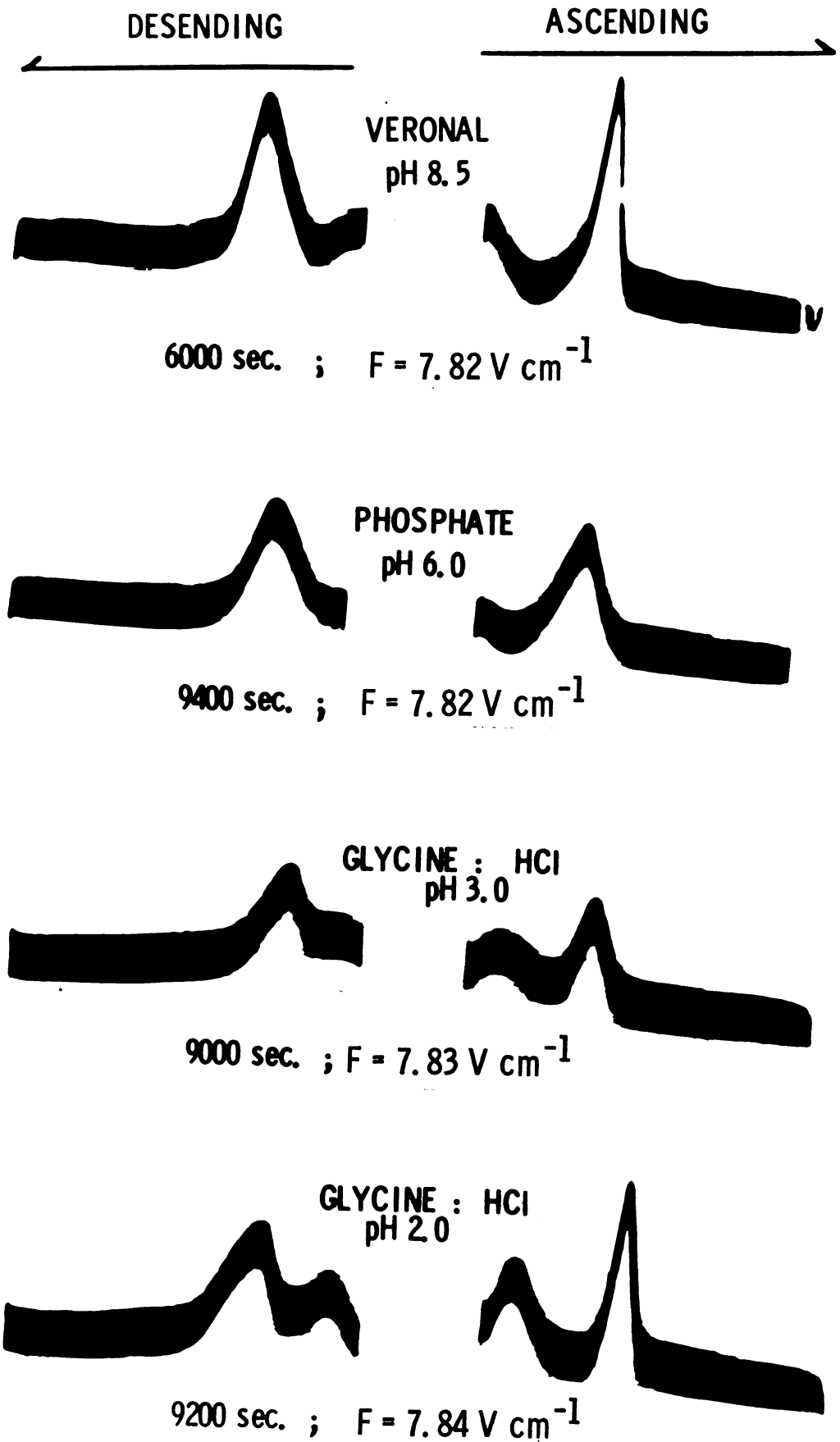


Figure 16. Free-boundary electrophoretic patterns of component 3 (heated preparation) in buffers of various pH.

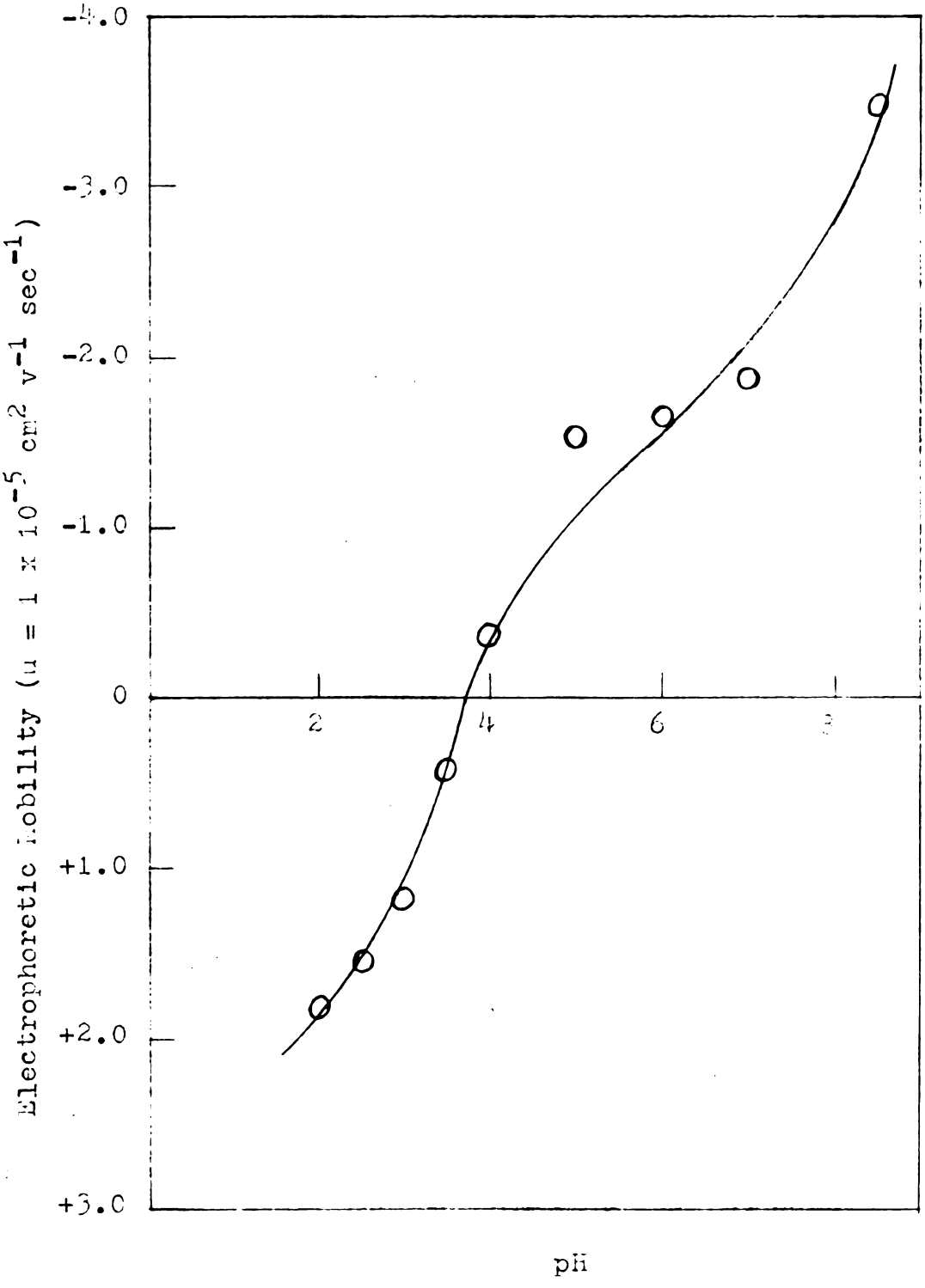


Figure 17. A plot of electrophoretic mobilities (descending values) of component 3 as a function of pH.

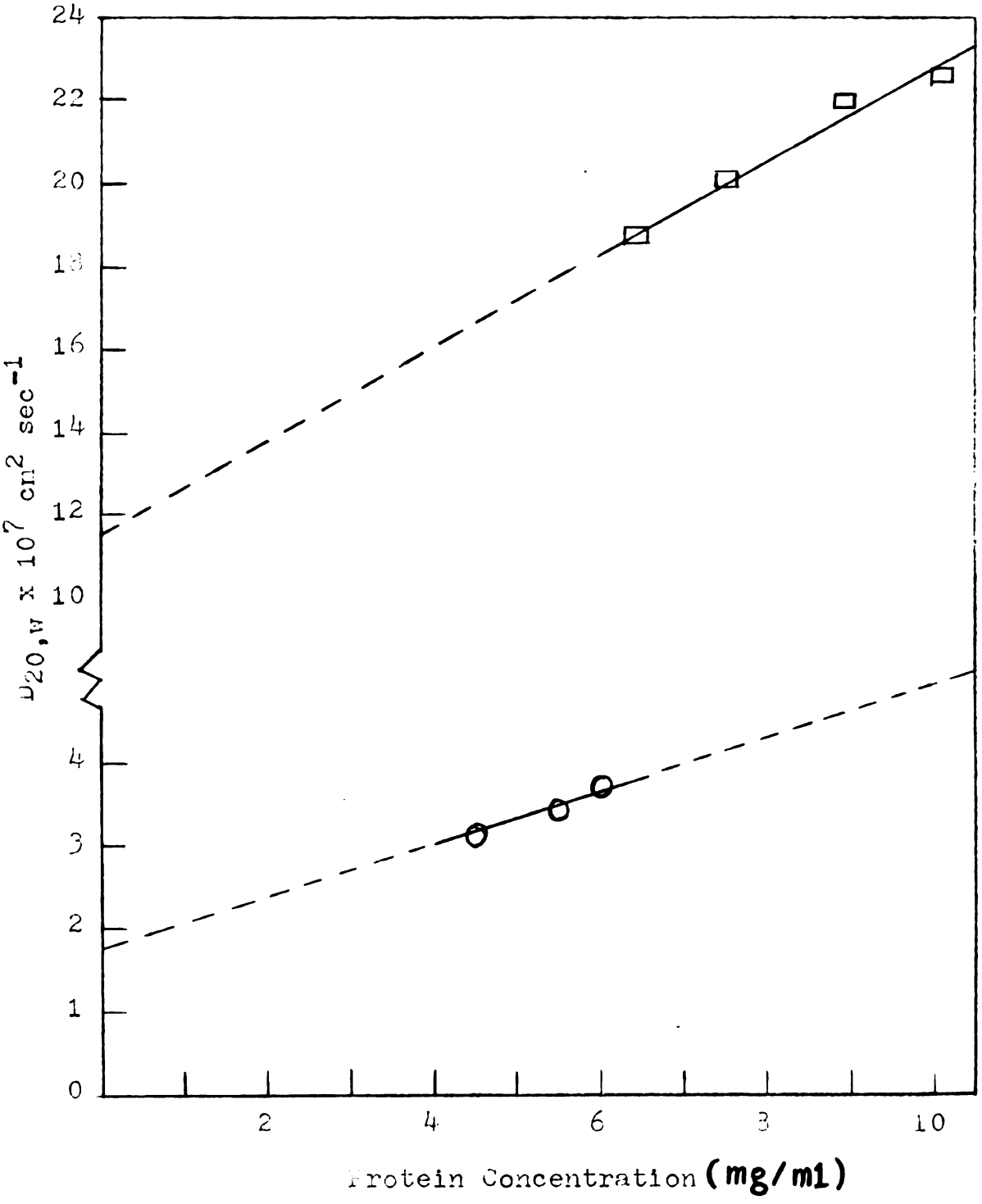


Figure 13. Plot showing concentration dependence of the apparent diffusion coefficient of component 3 (heated preparation) in veronal and veronal-5 M guanidine hydrochloride. Legend: O , veronal; □ , veronal-5 M guanidine hydrochloride.

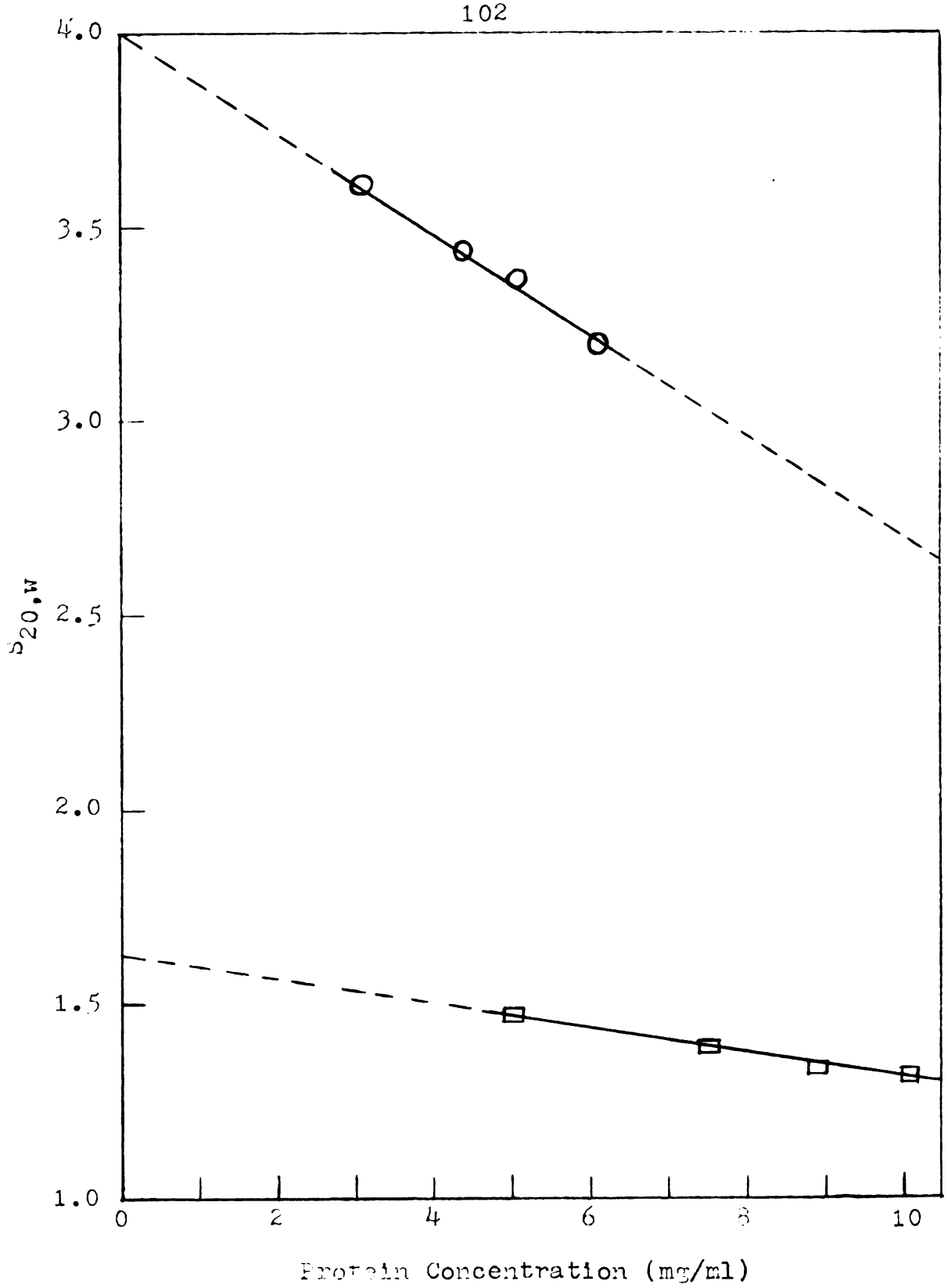


Figure 19. Plot showing the concentration dependence of the sedimentation coefficient of component 3 (heated preparation) in veronal and veronal-5 M guanidine hydrochloride buffer. Legend: O , veronal; □ , veronal-5 M guanidine hydrochloride.

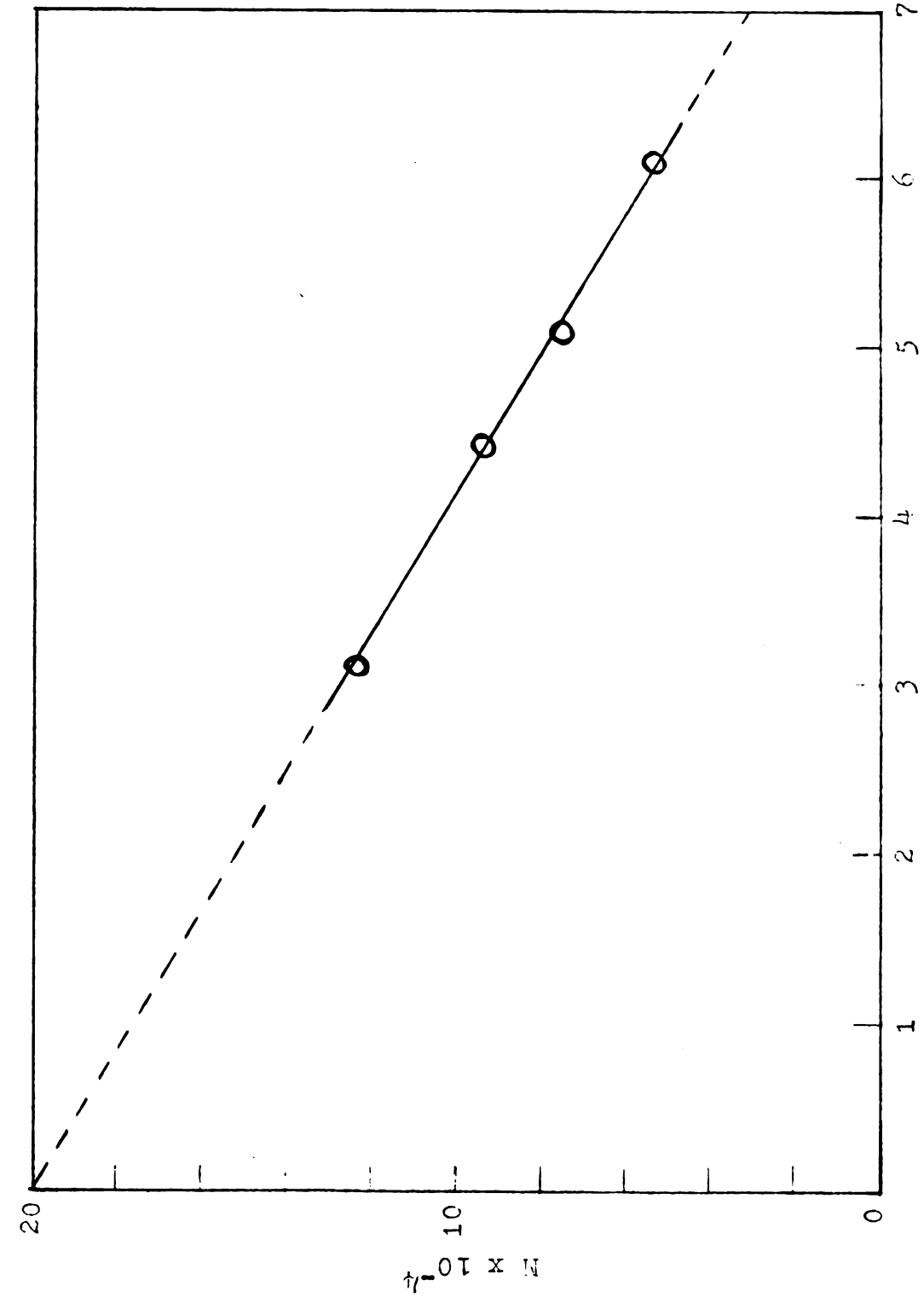


Figure 20. Plot showing concentration dependence of equilibrium molecular weight of component 3 (heated preparation) in veronal buffer.

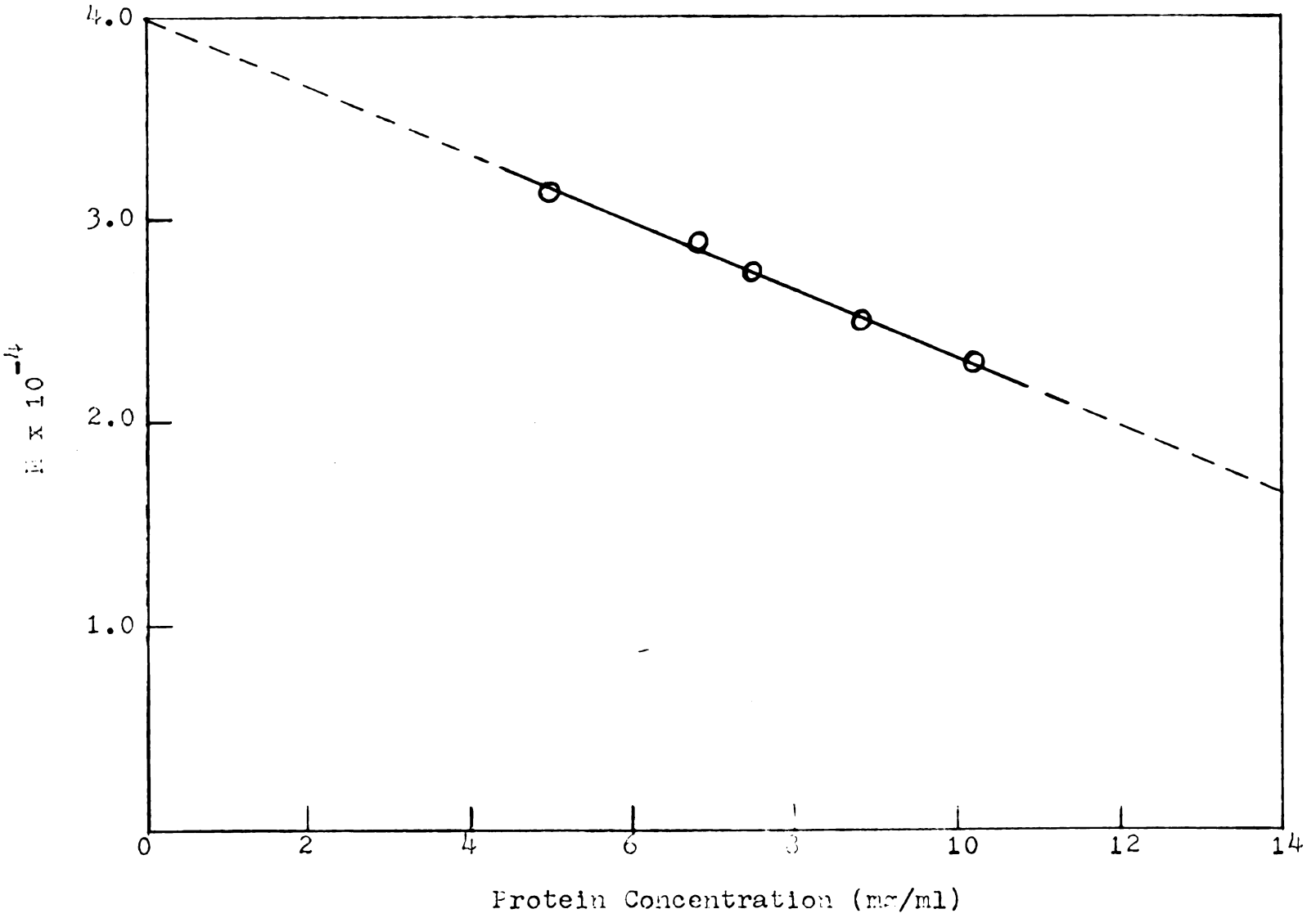
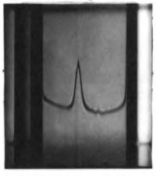


Figure 21. Plot showing concentration dependence of equilibrium molecular weight of component 3 (heated preparation) in veronal-5 M guanidine hydrochloride buffer.

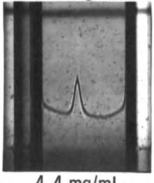
Figure 22. Sedimentation-velocity and sedimentation-equilibrium patterns for component 3 (heated preparation) in veronal buffer (pH 8.6; $\Gamma/2 = 0.1$). All experiments were performed at 20° C. The sedimentation-velocity patterns were taken at an elapsed time of 32 minutes, with the exception of the 3.1 mg/ml sample, which was taken at t = 16 minutes.

SEDIMENTATION-VELOCITY
59,780 RPM, $\theta = 55^0$

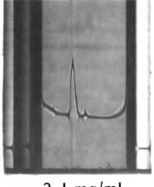
6.1 mg/ml



5.1 mg/ml

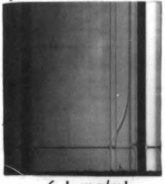


4.4 mg/ml

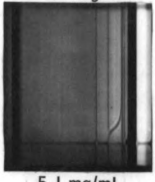


3.1 mg/ml

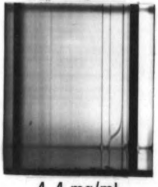
SEDIMENTATION-EQUILIBRIUM
11,573 RPM, $\theta = 60^0$



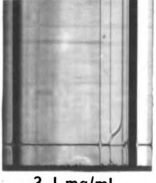
6.1 mg/ml



5.1 mg/ml



4.4 mg/ml



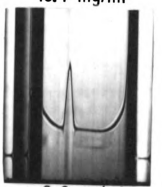
3.1 mg/ml

Figure 23. Sedimentation-velocity and sedimentation-equilibrium patterns for component 3 (heated preparation) in veronal-5 M guanidine hydrochloride buffer. All experiments were performed at 20° C. The sedimentation-velocity patterns were taken at the elapsed time of 32 minutes.

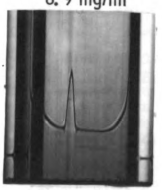
SEDIMENTATION-VELOCITY

59,780 RPM, $\theta = 55^0$

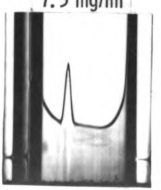
10.1 mg/ml



8.9 mg/ml



7.5 mg/ml

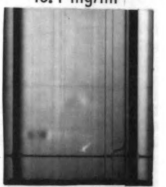


6.4 mg/ml

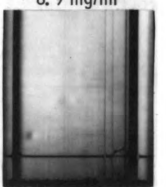
SEDIMENTATION-EQUILIBRIUM

9,341 RPM, $\theta = 60^0$

10.1 mg/ml



8.9 mg/ml



7.5 mg/ml

6.4 mg/ml

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APPENDIX

PART I

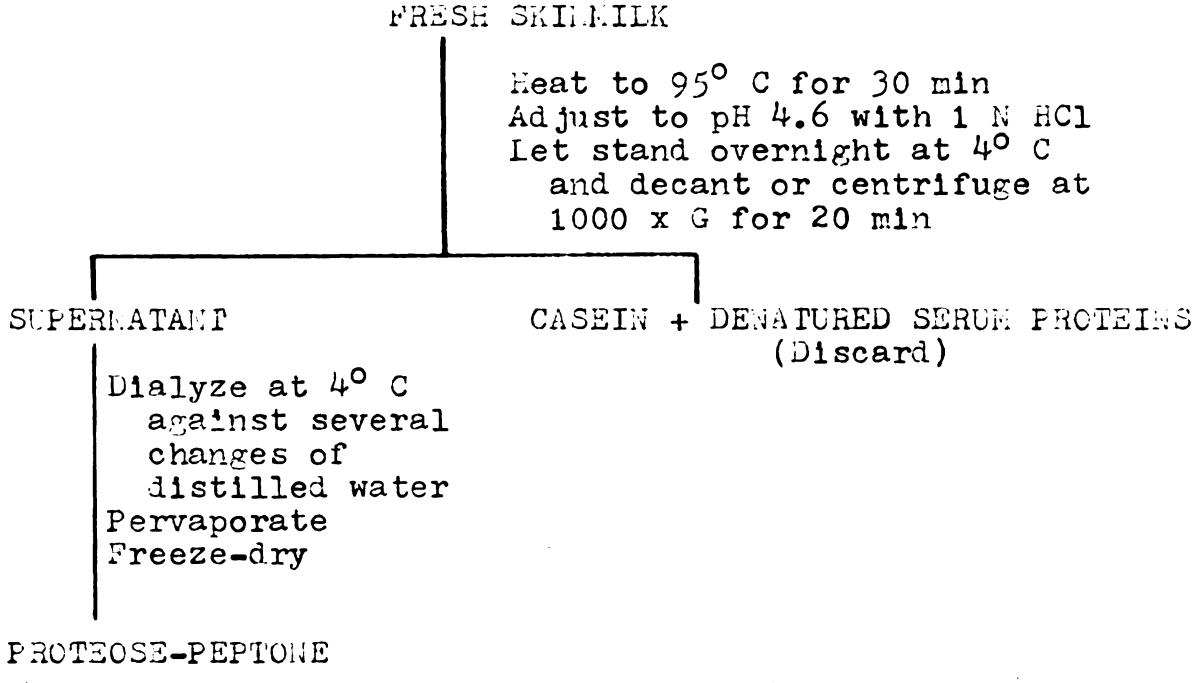

Figure I. Preparation of the classical proteose-peptone fraction.

Composition of the Buffers Employed in This Study

The following buffer preparations were used in testing the effect of buffer pH on the hexose content of purified component 3:

1. Citrate phosphate; pH 4.6

   A = 0.1 M solution of citric acid (19.21 g in 1000 ml)

   B = 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4 \cdot 7H_2O$ or 71.7 g of $Na_2HPO_4 \cdot 12H_2O$ in 1000 ml)

   26.7 ml of A + 23.3 ml of B, diluted to a total of 100 ml

2. Citrate phosphate; pH 7.0

   Same stocks buffers as A and B above.

   6.5 ml of A + 43.6 ml of B, diluted to a total of 100 ml

3. Tris citrate; pH 8.60

   Same as gel buffers used in acrylamide gel electrophoresis and starch-urea gel electrophoresis

   Stock solution = 91.96 g Tris (solid) + 12.05 g citric acid, diluted to a total of 100 ml

   Use one part stock to nine parts deionized water

4. Borate; pH 8.60

   Same as buffer used in electrode compartments in acrylamide gel electrophoresis and starch-urea gel electrophoresis.

   Stock solution = 881 g boric acid + 190 g NaOH, diluted to a total of 19 liters

   Use two part stocks to three parts deionized water

Formulation for Starch-Urea Gel

Ingredients: 35 ml Tris stock solution; 205 ml distilled water, 40 g starch and 147 g urea. Heat Tris stock, water and starch to 65° C with stirring, add urea, heat rapidly with stirring to 90° C, degas and pour into gel bed.

Preparations of Discontinuous Buffers, Staining Solution and Destaining Solution for Preparative Acrylamide Gel Electrophoresis and Starch-Urea Gel Electrophoresis

Buffers:

Tris citrate, pH 8.6

Stock solution = 91.96 g of Tris (solid) + 12.05 g citric acid, diluted to a total of 1000 ml

Use one part stock to nine parts water

Borate, pH 8.6

Stock solution = 881 g boric acid + 190 g NaOH, diluted to a total of 19 liters

Use two parts stock to three parts water

Staining solution:

250 ml water, 250 ml methanol, 50 ml glacial acetic acid and 20 amino or buffalo black

Washing solution:

200 ml glycerin, 1 liter water, 1 liter methanol, 200 ml glacial acidic acid for mechanical destaining. 7% acetic acid for electrolytic destaining

Reagents Used in Orcinol-Sulfuric Acid Method

Reagent A = 60 ml of concentrated sulfuric acid and 40 ml water

Reagent B = 1.69 ml of orcinol (recrystallized from benzene) in 100 ml water

7.5 ml reagent A is mixed with 1 ml of reagent B

Reagents Used in Folin-Ciocalteu Colorimetric Reaction

Reagent A = 2% Na_2CO_3 in 0.1 N NaOH

Reagent B = 0.5% $CuSO_4 \cdot 5H_2O$ in 1% Na or K tartrate

Reagent C = mix 50 ml A with 1 ml B

Reagent D = Folin-Ciocalteu reagent diluted with 1 N HCl

APPENDIX

PART II

Standard Curves for the Colorimetric Determinations of Phosphorus, Tryptophan, Cystein, Hexose, Hexosamine and Sialic Acid Are Given Below

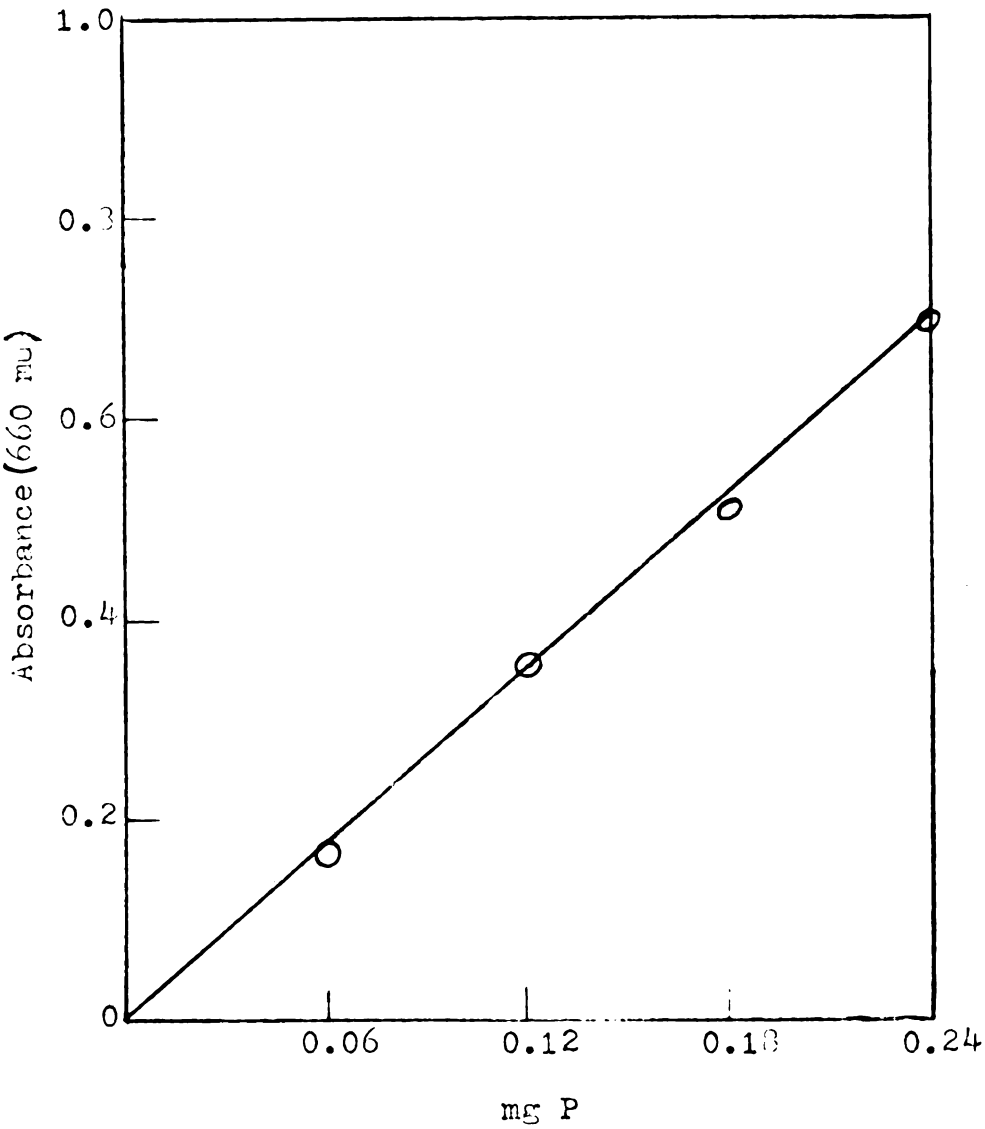

Figure II. Standard curve for phosphorus.

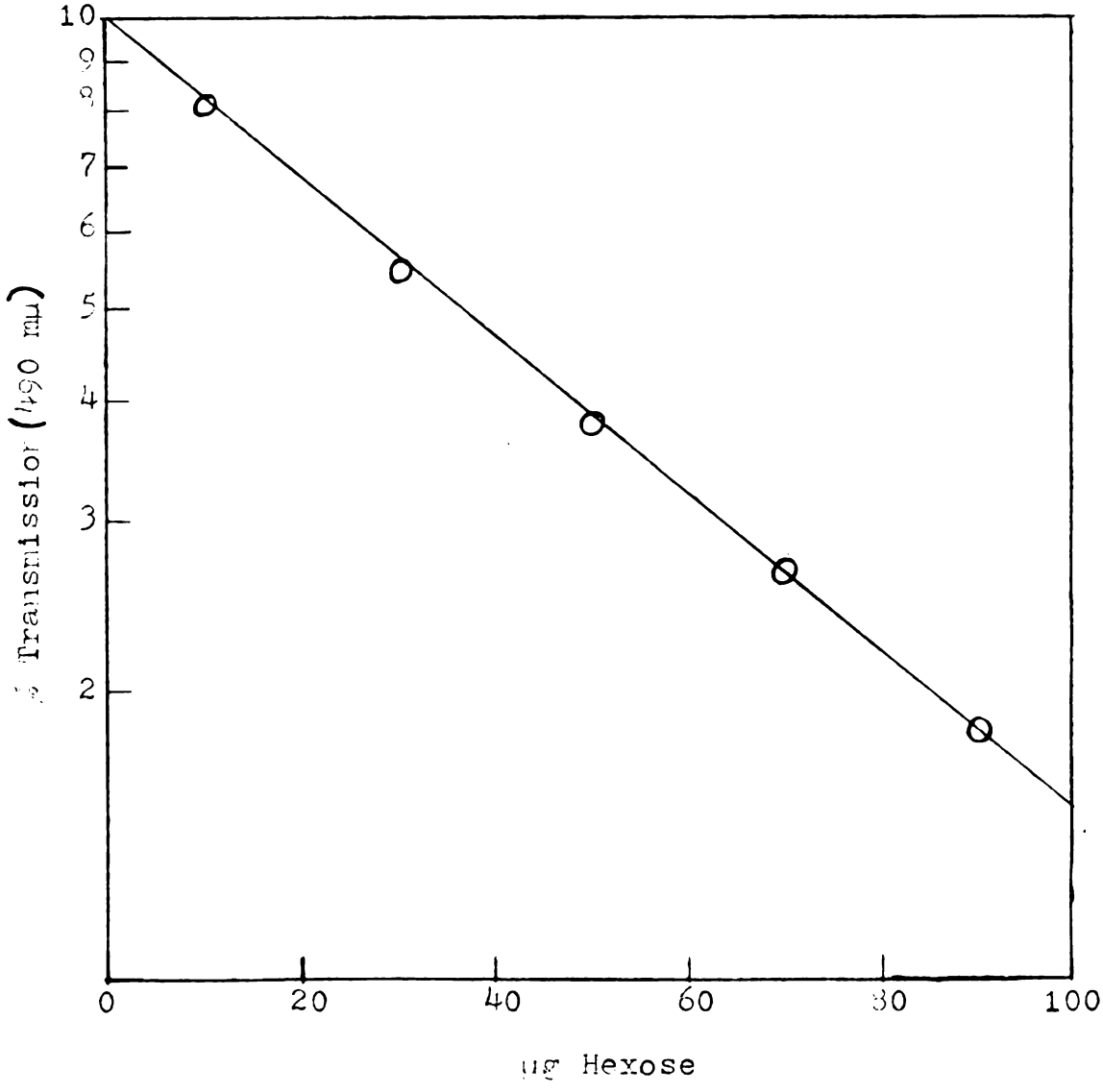

Figure III. Standard curve for galactose.

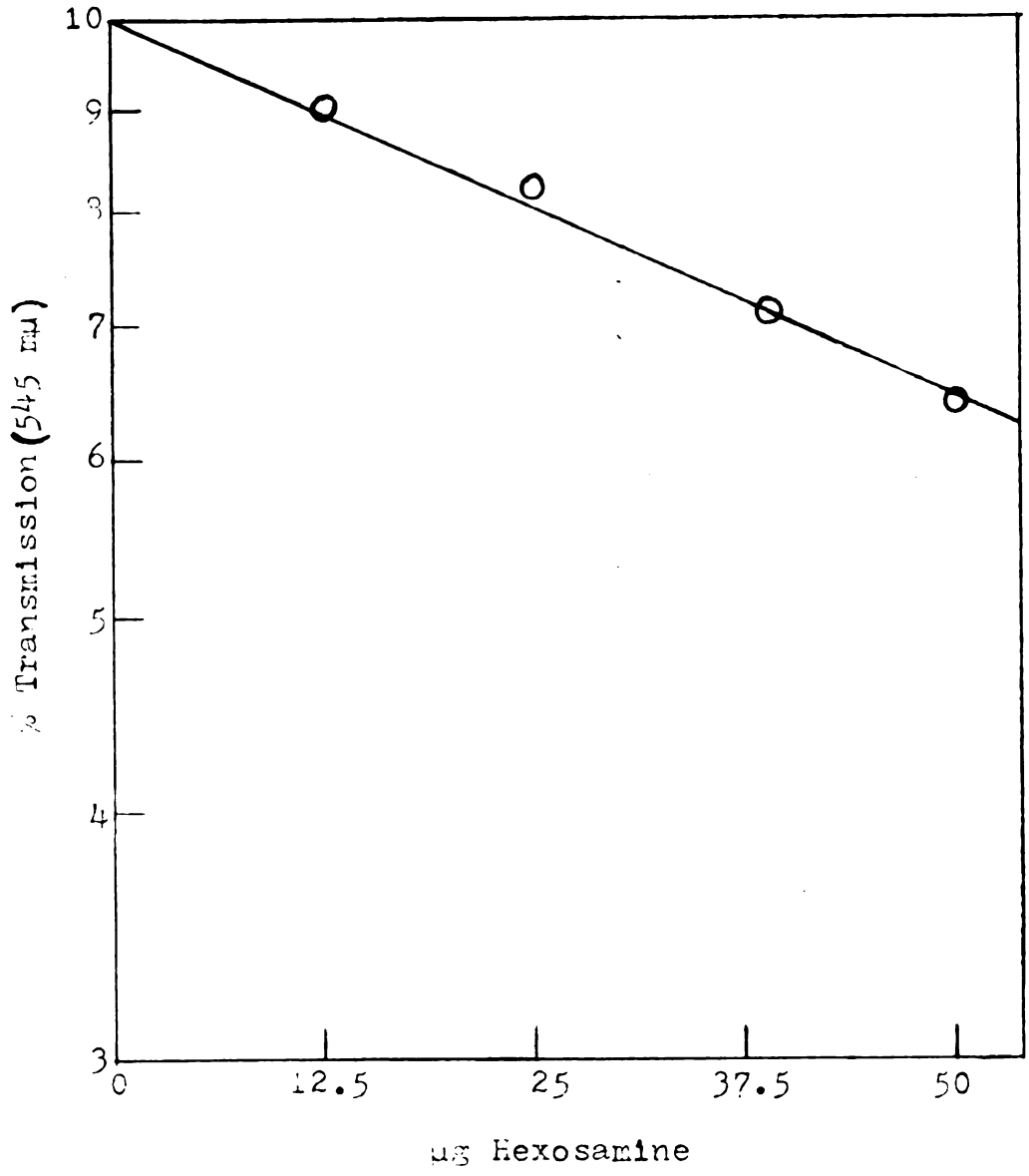

Figure IV. Standard curve for glucosamine-HCl.

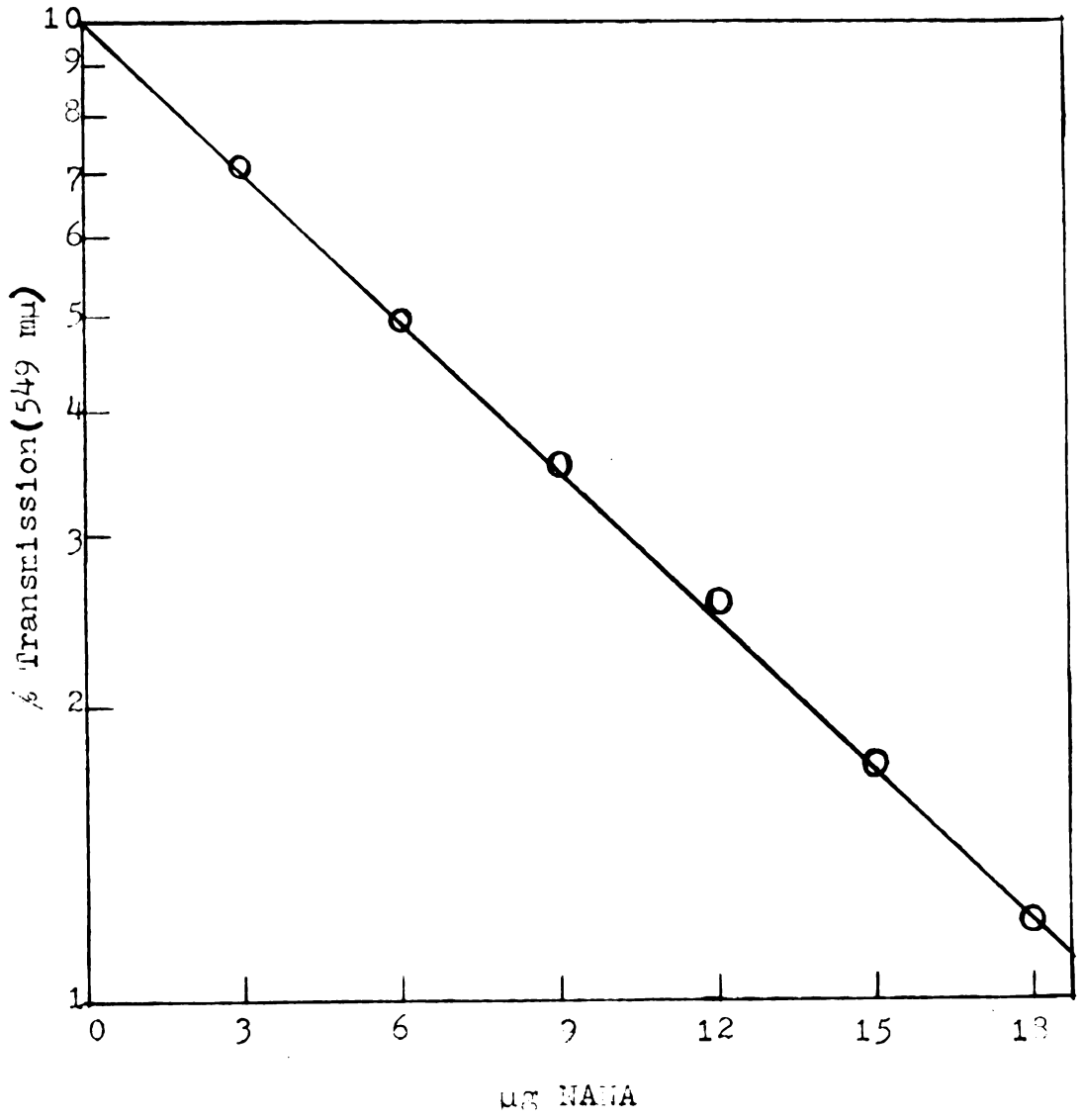

Figure V. Standard curve for N-acetylneuraminic acid (synthetic).

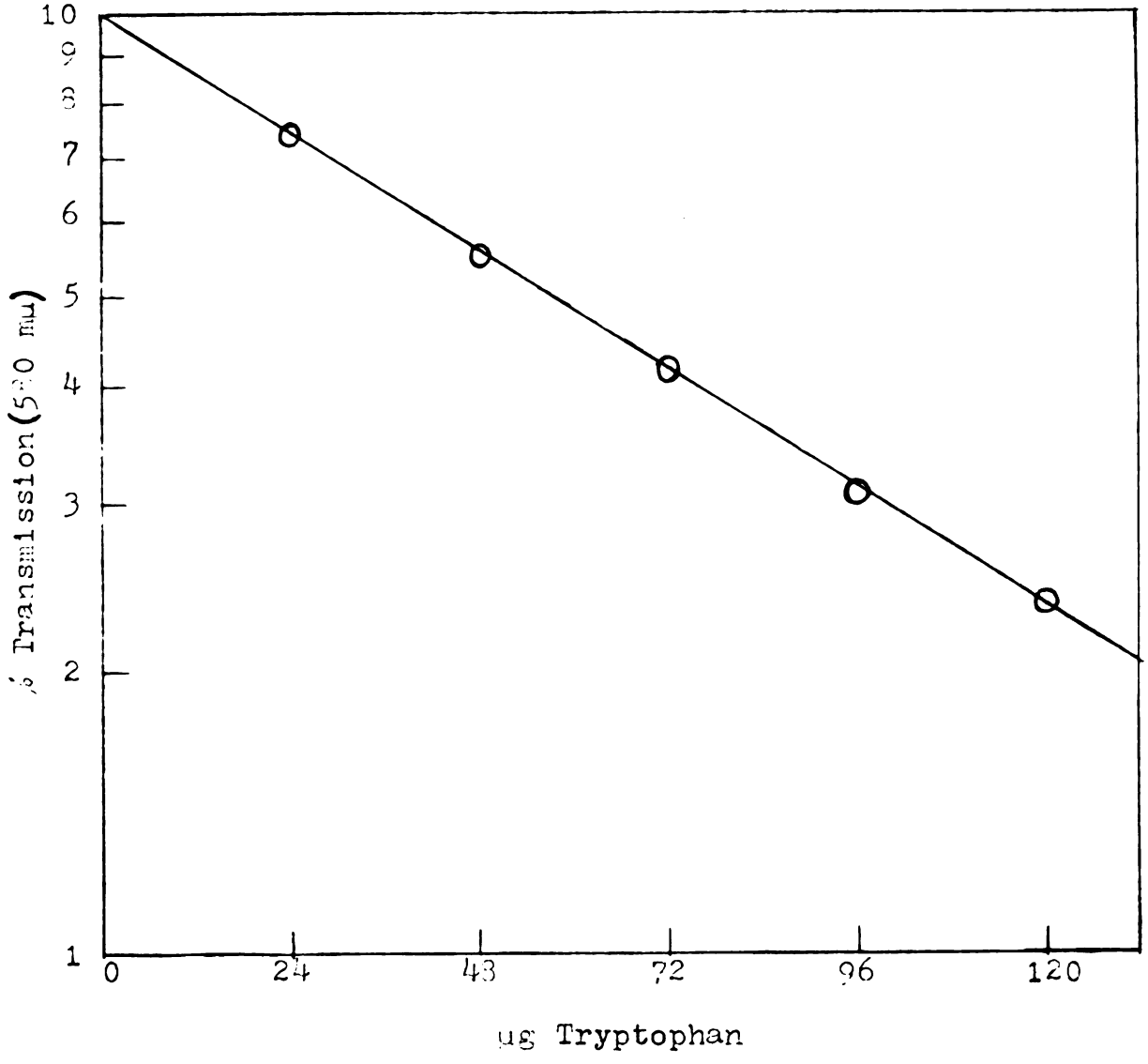

Figure VI. Standard curve for tryptophan.

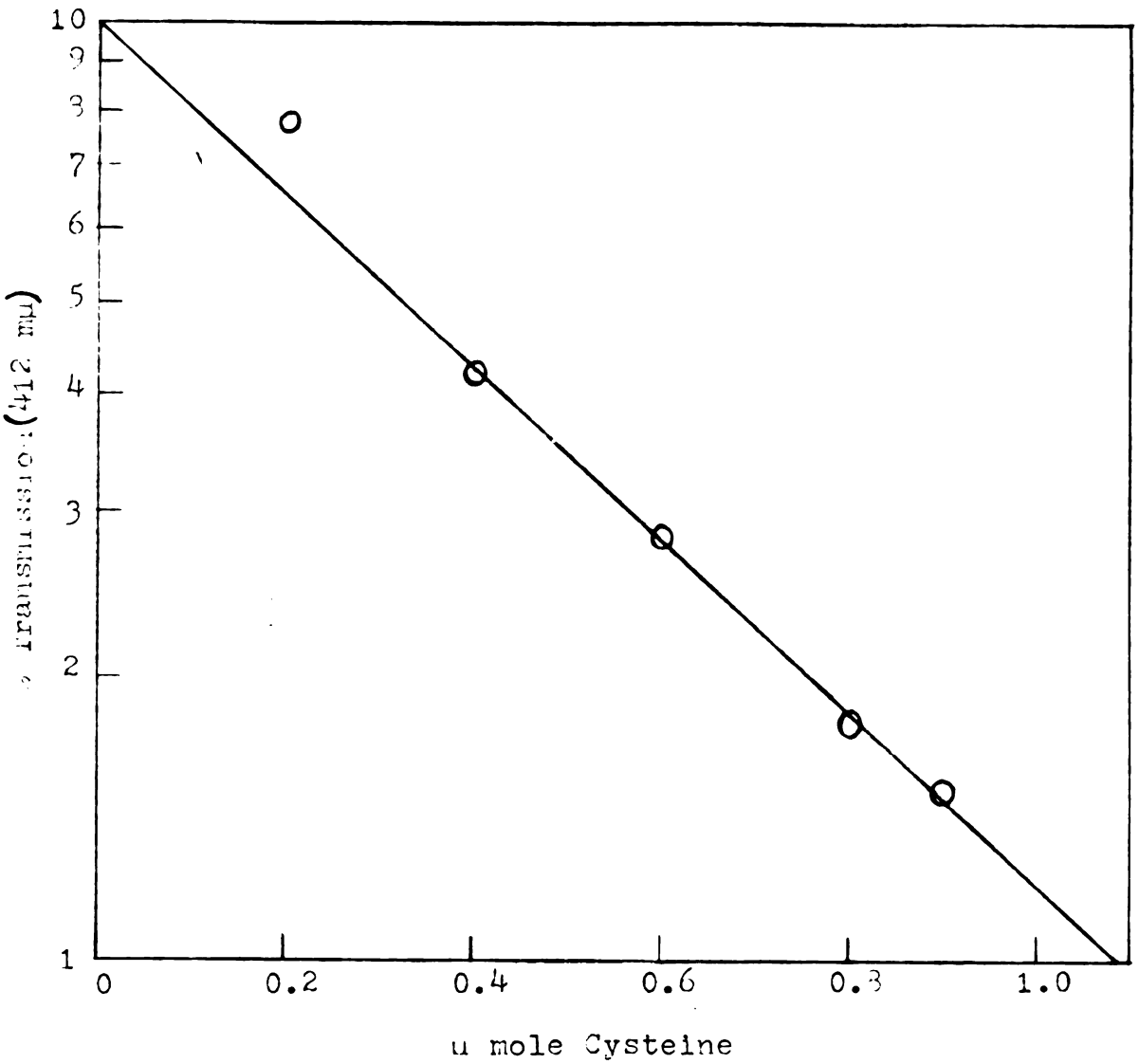

Figure VII. Standard curve for cysteine.

Buffer Preparations Used in Free-Boundary Electrophoresis

The following quantities were made to 2 liters with redistilled water:

1. Sodium veronal; pH 8.6; $\Gamma/2 = 0.2$

   72.0 ml of 5.0 M NaCl

   3.5 ml of 2.0 M HCl

   30.0 ml of 0.5 M sodium veronal

2. Sodium phosphate; pH 7.0; $\Gamma/2 = 0.2$

   72.0 ml of 5.0 M NaCl

   22.7 ml of 0.5 M Na_2HSO_4

   1.6 ml of 4.0 M NaH_2PO_4

3. Sodium phosphate; pH 6.0; $\Gamma/2 = 0.2$

   72.0 ml of 5.0 M NaCl

   9.2 ml of 0.5 M Na_2HPO_4

   6.6 ml of 4.0 M NaH_2PO_4

4. Sodium Acetate; pH 5.0; $\Gamma/2 = 0.2$

   72.0 ml of 5.0 M NaCl

   20.0 ml of 2.0 M NaAc

   3.7 ml of 3.5 M acetic acid

5. Sodium acetate; pH 4.0; $\Gamma/2 = 0.2$

   72.0 ml of 5.0 M NaCl

   20.0 ml of 2.0 M NaAc

   33.7 ml of 3.5 M acetic acid

6. Glycine - HCl; pH 3.5; $\Gamma/2 = 0.2$

   72.0 ml of 5.0 M NaCl

   36.6 ml of 1.0 M glycine - 1.0 M NaCl

   1.7 ml of 2.0 M HCl

7. Glycine - HCl; pH 3.0; $\Gamma/2 = 0.2$

72.0 ml of 5.0 M NaCl

31.6 ml of 1.0 M glycine - 1.0 M NaCl

4.2 ml of 2.0 M HCl

8. Glycine - HCl; pH 2.5; $\Gamma/2 = 0.2$

72.0 ml of 5.0 M NaCl

22.3 ml of 1.0 M glycine - 1.0 M NaCl

8.6 ml of 2.0 M HCl

9. Glycine - HCl; pH 2.0; $\Gamma/2 = 0.2$

72.0 ml of 5.0 M NaCl

10.6 ml of 1.0 M glycine - 1.0 M NaCl

14.7 ml of 2.0 M HCl

Composition of Veronal Buffer Used in Ultracentrifugal and Diffusion Runs

Veronal; pH 8.6; $\Gamma/2 = 0.1$

20.6 g sodium barbiturate

2.797 gm barbituric acid

Made up to a total of 1 liter with redistilled water

Properties of the Solvents Used for Molecular Weight Calculations and Correction of the Sedimentation and Diffusion Coefficients to Water

TABLE I

Densities and relative viscosities of some of the solvents

Solvent	η_{o}/η_{w}	$\rho_{20^\circ C}$
Veronal buffer	1.101	1.1034
Veronal - 5 M guanidine HCl buffer	1.437	1.1215

Correction of the Observed S to Standard Conditions

The experimentally determined sedimentation coefficient was corrected to a value corresponding to sedimentation coefficient in water at 20° C. The equation commonly used is

$$S_{20,w} = \left(\frac{\eta_{w,t}}{\eta_{w,20}}\right)\left(\frac{\eta_{o,t}}{\eta_{w,t}}\right)\left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{t,o}}\right) S_T$$

where the first term is the viscosity of water at the experimental temperature relative to that at 20° C, the second term is the relative viscosity of the solvent to that of water, and the last term is the relative bucyancy term containing the partial specific volume, the density of water at 20° C, and the density of the solution at the experimental temperature.

Correction of the Observed Diffusion Coefficient to Standard Conditions

The experimentally determined diffusion coefficient was corrected to a value corresponding to diffusion coefficient in water at 20° C. The equation used is

$$D_{20,w} = \left(\frac{293}{273 + t}\right)\left(\frac{\eta_{o,t}}{\eta_{w,t}}\right)\left(\frac{\eta_{w,t}}{\eta_{w,20}}\right) D_T$$

where D_T is the experimentally measured diffusion coefficient at temperature t, t is the temperature of the diffusion experiment in degree centigrade, $(\eta_{o,t}/\eta_{w,t})$ is the relative viscosity of the solvent to that of water, $\eta_{w,t}$ is the viscosity of water at the temperature of the experiment, and $\eta_{20,w}$ is the viscosity of water at 20° C.

Calculation of partial specific volume

The partial specific volume was calculated from the amino acid residue weights and the weight percentages of the carbohydrate components, according to the following equations,

$$\bar{v} = \frac{\sum v_i w_i}{\sum w_i}$$

where $\bar{v}$ = partial specific volume of the protein

v_i = partial specific volume of the i^{th} component

w_i = weight of the i^{th} component

A sample tabulation if shown on the next page. By this method, a $\bar{v}$ of 0.715 was obtained, e.g.,

$$\bar{v} = \frac{\sum v_i w_i}{\sum w_i} = \frac{67.45}{95.35} = 0.715$$

TABLE III

Data and sample calculation of partial specific volume of component 3

Components	W_i	V_i	V_iW_i
Lysine	7.31	0.82	5.99
Histidine	3.27	0.67	2.19
Arginine	4.04	0.70	2.83
Unidentified peak	1.79	0.70	1.25
Aspartic acid	6.47	0.60	3.88
Threonine	5.58	0.70	3.91
Serine	6.26	0.63	3.94
Glutamic acid	14.40	0.66	9.50
Proline	4.79	0.76	3.64
Glycine	1.07	0.64	0.69
Alanine	2.16	0.74	1.60
Unidentified peak	0.27	0.86	0.23
Valine	2.16	0.86	1.86
Methionine	1.60	0.75	1.20
Isoleucine	4.82	0.90	4.33
Leucine	8.15	0.90	7.34
Tyrosine	0.74	0.71	0.53
Phenylalanine	2.28	0.77	1.76
Hexose	8.20	0.613	5.03
Hexosamine	6.00	0.666	4.00
Sialic acid	3.00	0.584	1.75
	$\Sigma W_i = 94.36$		$\Sigma V_iW_i = 67.45$

Sedimentation Equilibrium Method for Molecular Weight Determination

The method used in the calculation of equilibrium weight-average molecular weight from ultracentrifugal data is presented here. The equations involved and sample calculations are shown. For a thorough and extensive treatment of the theory, the reader is referred to the following references: Schachman (1959); Tanford (1961); Williams, Van Holde (1960); Trautman (1956); Svedberg and Peterson (1940); and Wales (1961).

Notation

The following symbols appear in this section:

- c - Concentration in gm/ml
- c_m - Concentration of protein at the air-liquid meniscus
- c_b - Concentration of protein at the bottom of the solution
- c^o - Initial concentration
- D - Diffusion coefficient
- M_o - Magnification of the camera lens (radial)
- R - Gas constant (8.314×10^7 ergs deg^{-1} $mole^{-1}$)
- r - Distance from the center of rotation
- r_b - Distance from the center of rotation to the bottom of the solution
- r_m - Distance from the center of rotation to the air-liquid meniscus
- S - Sedimentation coefficient
- T - Absolute temperature

t - time

$\bar{v}$ - Partial specific volume of solute

X - M_o r

X_b - M_o r_m

X - Radial distance between measurements on the photographic plate

Y - Vertical distance between the solvent and solution pattern

ρ - Density

ω - Angular velocity of the rotor (radians sec^{-1})

Example Calculation

The microcomparator readings and the calculations for the equilibrium weight-average molecular weight are presented in this section. The symbol Rn refers to the distance from the inner reference line to the designated position as measured by the microcomparator on the photographic plate. For the Model E used, r_{inner} = 5.72 cm at low speeds and M_o = 2.103

TABLE III

Data and example calculation from a short-column equilibrium pattern

Protein = Component 3 from heated skimmilk
Speed = 9,341 RPM Conc. = 5.0 mg/ml
Temperature = 20.0° C Buffer = Veronal-5 M guanidine-HCl

n	Rn	Yn	Yn
0	3.032	0.361*	
1	3.022	0.357	0.357
2	3.002	0.348	0.705

(Continuation of Table III)

3	2.932	0.340	1.045
4	2.962	0.332	1.377
5	2.942	0.326	1.703
6	2.922	0.320	2.023
7	2.902	0.314	2.337
8	2.882	0.307	2.644
9	2.862	0.301	2.945
10	2.842	0.295	3.240
11	2.822	0.289	3.529
12	2.802	0.283	3.812
13	2.782	0.276	4.083
14	2.762	0.269	4.357
15	2.742	0.263	4.620
16	2.722	0.257	4.877
17	2.702	0.252	5.129
18	2.709	0.251*	

$R_b = 3.032$ $\quad r_b = \frac{R_b}{2.103} + 5.72 = 7.162$ $\quad r_b^2 = 51.294$

$R_m = 2.709$ $\quad r_m = \frac{R_m}{2.103} + 5.72 = 7.008$ $\quad r_m^2 = 49.112$

*Values obtained by extrapolation

$$c_b - c_m = \frac{X}{K_o} \sum_{1}^{17} Y_n, \quad \Delta Y_{zero} = 0.142$$

$$c_b - c_n = \frac{0.02}{2.103} \left[5.129 - (0.142) \times 17 \right]$$

$$= \frac{0.02}{2,103} \times 2.715$$

$$= 0.0258$$

c_o is obtained by similar measurements of a synthetic boundary pattern obtained at the same schlieren diaphragm angle, i.e.

$$c_o = \frac{\Delta X}{K_o} \sum \Delta Y_{\text{syn. boundary}} = 0.1051$$

Other quantities necessary for the calculations are:

$$\rho_{solution} = 1.1215$$

$$\frac{RT}{(1 - \bar{v}\rho)W^2} = \frac{(8.314 \times 10^7)(293)}{(1 - 0.715 \times 1.1215)(0.97535 \times 10^6)}$$

$$= 1.2368 \times 10^5$$

$$\frac{1}{r_b^2 - r_m^2} = 0.4533$$

a) weight-average molecular weights for the total cell contents is calculated from the following equation,

$$M_w^{app} = 2\frac{RT}{(1 - \bar{v}\rho)W^2} \times \frac{c_b - c_m}{c^o} \times \frac{1}{r_b^2 - r_m^2}$$

$$= 2\,(1.2368 \times 10^5)\left(\frac{0.0253}{0.1051}\right)(0.4533)$$

$$\cong 28{,}960$$

b) Z-average molecular weight for the total cell contents,

$$M_z^{app} = \frac{RT}{(1 - \bar{v}\rho)W^2}\left(\frac{\frac{Y_b}{r_b} - \frac{Y_m}{r_m}}{c_b - c_m}\right)$$

$$= (1.2368 \times 10^5)\left(\frac{\frac{0.1717}{7.162} - \frac{0.1194}{7.008}}{0.0253}\right)$$

$$\cong 34{,}600$$

Calculation of Molecular Weights from the Sedimentation Diffusion Data

Molecular weights can be calculated directly from the sedimentation and diffusion coefficients, at infinite dilution, according to the Svedberg equation,

$$M = \frac{RTs}{D\,(1 - \bar{v}\rho)}$$

Here, $R = 8.314 \times 10^{7}$ ergs deg^{-1} $mole^{-1}$

$T = 293^{o}$

$s = 4.0 \times 10^{-13}$ sec (at infinite dilution, in veronal buffer)

$D = 1.8 \times 10^{-7}$ cm^{2}/sec (at infinite dilution, in veronal buffer)

$\bar{v} = 0.715$

$\rho = 1.034$

for component 3 from heated skimmilk, in veronal buffer, the molecular weight calculated from the above equation is,

$$M_{S,D} = \frac{(8.314 \times 10^{7})\ (293)\ (4.0 \times 10^{-13})}{(1.8 \times 10^{-7})\ \ (1 - 0.715 \times 1.034)}$$

$$= 207{,}000$$

This value agrees satisfactorily with a molecular weight of 200,000 of component 3, at infinite dilution, in same veronal buffer, from sedimentation-equilibrium data.