INTERRELATION OF BOVINE SERUM PROTEINS AND MILK PROTEOSE-PEPTONE COMPONENTS

Thesis for the Degree of M. S.

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JUNG JA KANG

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ABSTRACT

INTERRELATION OF BOVINE SERUM PROTEINS
AND MILK PROTEOSE-PEPTONE COMPONENTS

By

Jung Ja Kang

The proteose-peptone fraction of bovine milk is designated as a group of heat-stable, acid-soluble glycoproteins. This fraction accounts for approximately 18 to 25% of the whey protein or 4% of the total proteins in bovine milk. Both proteose-peptone and a heat-stable fraction from bovine serum were prepared. The latter fraction was considered to be analogous to the proteose-peptone fraction in whey.

Acrylamide gel electrophoretic analysis of these preparations was performed with both continuous and discontinuous buffer systems. Component 3 of proteose-peptone migrated as a single zone in both systems. Component 5 which appeared as a single zone by the continuous buffer system was resolved into five closely migrating zones by electrophoresis in a discontinuous system. Component 8 appeared as two closely migrating zones in gels obtained with the discontinuous system, but was

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separated into two multi-zoned areas, component 8-fast and 8-slow, in gels obtained with the continuous system.

Electropherograms of heated bovine serum and proteose-peptone preparations indicated the possibility that these preparations contained some common components.

Carbohydrate-containing components of proteose-peptone and heat-stable bovine serum were identified in acrylamide gels by the periodic acid-Schiff technique. The principal components of the proteose-peptone preparation--3, 5, and 8--were identified as glycoproteins. Only one zone in the heat-stable serum preparation, possessing the same relative mobility as proteose-peptone component 5, showed a positive reaction with the periodic acid-Schiff glycoprotein reagent.

Immunological assays were performed, using hyper-immunized rabbit anti-sera against proteose-peptone and bovine serum, by immuno-doublediffusion or immunoelectrophoresis. A proteose-peptone preparation, as well as purified components 3, 8-slow and 8-fast, a heat-stable serum preparation, and a protein preparation obtained from the fat globule membrane were employed as antigens. Whey and bovine serum served as reference systems. These experiments demonstrated the existence of some components common to proteose-peptone and bovine serum, as well as to proteose-peptone and the membrane protein. The proteose-peptone preparation contained additional

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components which were not of serum origin. Proteose-peptone components 3 and 8-slow showed immunological reactions with anti-bovine serum. The results obtained with components 5 and 8-fast were inconclusive and should be further investigated.

INTERRELATION OF BOVINE SERUM PROTEINS AND MILK PROTEOSE-PEPTONE COMPONENTS

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Jung Ja Kang

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INTRODUCTION

The proteose-peptone fraction of bovine milk is designated as a group of heat-stable, acid-soluble glycoproteins. The principal components of this minor protein fraction are characterized as component "3," "5," and "8" in free-boundary electrophoretic patterns. Component 8 is further fractionated into components 8-fast and 8-slow by gel filtration on Bio-Gel P-10. The classical isolation method for proteose-peptone consists of heating to 96-100 C for 20 min followed by acidification to pH 4.6 to remove all principal milk proteins.

A distribution study of proteose-peptone components in the milk system revealed that component 3 was not found in micellar casein, whereas component 5 and 8 were found both in whey and micellar casein. This observation led to the suggestion that component 3 might be of serum origin. Previous studies in other laboratories demonstrated that about six of fifteen identifiable whey proteins were of serum origin. Chemical compositional data of proteose-peptone indicate that some similarity with serum proteins exist. Some indications that the phospho-glycoproteins of milk and colostrum are similar to serum glycoproteins have been reported. Thus, it is predicted that some components,

if not all, of the proteose-peptone fraction might originate from bovine serum.

The purpose of this study was to determine if the proteose-peptone components of milk and especially component 3 originate from the serum. The technique of acrylamide gel electrophoresis and immunological assay were employed for this purpose.

LITERATURE REVIEW

Osborne and Wakeman (1918) were among the early workers who suggested the presence of a heat-stable and acid-soluble protein in milk. They noted that variable amounts of proteinaceous material remained in solution following the heat denaturation of albumins and globuline in acid whey. However they did not conclude whether or not the proteose-peptone was an original constituent of milk or an artifact resulting from the heat treatment.

Palmer and Scott (1919) claimed that lactalbumin and lactoglobulin did not represent all of the protein in the casein-free filtrate because it was possible to recover protein from the heat-coagulated filtrate by the addition ot tannic acid.

Kieferle and Gloetzl (1930), and Kieferle (1933) recovered the soluble proteins which were not coagulable by heat by the addition of phosphotungstic acid; these were classified as proteose and peptone materials.

Jones and Little (1933) reported the presence of considerable amounts of proteose in milk. The proteins precipitated by 5% trichloracetic acid were classified as whole protein while the material precipitated by 10% trichloracetic acid was classified as protein-like proteose.

Rowland (1938a) designated the proteose-peptone as a protein fraction which did not precipitate at pH 4.7 after heating for 30 min but was precipitated by 12% trichloracetic acid. He concluded that the soluble protein fraction of normal fresh milk (whey) is composed of approximately 76% albumin and globulin, and 24% proteose-peptone substances. He (1937) also found that the non-protein nitrogen content was not affected by heating up to 100 C and that on continued heating at 95 and 100 C only small amounts of proteose resulted from the hydrolysis of protein. Later he (1938b) reported the nitrogen distribution of normal milk as follows: 18.5% casein N, 9.2% albumin N, 3.3% globulin N, 4.0% proteose-peptone N and 5.0% non-protein N.

Harland and Ashworth (1945) observed that casein obtained by saturating skimmilk with NaCl accounted for more nitrogen than could be attributed to the casein, whereas the whey protein nitrogen content was lower than that released by Rowland's acid precipitation method. Ninety-five per cent of the whey proteins in this preparation were coagulable by severe heat treatment compared with only 76% by Rowland's method.

Aschaffenburg (1946) fractionated a surface active material, which he called sigma proteose, by salting-out of a heated acid or rennet whey with ammonium sulfate after heating skimmilk to 90-95 C for 15 min. This

fraction was completely insoluble in 50% saturated ammonium sulfate.

Ogston (1946) examined this fraction by ultra-centrifugation and found that it was heterogeneous with one-half of the material consisting of a single, well defined constituent.

Weinstein, Duncan and Trout (1951) isolated a minor protein fraction from heated rennet whey which was capable of producing a solar-activated flavor in homogenized milk. This fraction was different from Aschaffenburg's fraction in elemental analysis and amino acid composition.

Ashworth and Drueger (1951) studied the nitrogen distribution in milk, comparing three different methods: the official A.O.A.C. method, Rowland's method and the NaCl- precipitation method of Harland and Ashworth. The NaCl precipitation method was characterized by being highest in casein-N and lowest in proteose-peptone N. The whey and non-protein nitrogen contents were similar by all three methods. They suggested that the proteose-peptone described by Rowland might be the small molecular weight species of casein remaining as soluble protein at its isoelectric point.

Larson and Rolleri (1955) attributed the three peaks--"3," "5," and "8"--in moving-boundary electrophoretic patterns of heat-treated acid whey to the proteose-peptone fraction of milk. They also designated

other milk serum proteins from heated and unheated milk, i.e., euglobulin, pseudoglobulin, α-lactalbumin, β-lactglobulin and serum albumin, as components 1, 2, 4, 6, and 7, respectively. Further, they suggested that the proteose-peptone was a native component in milk since it was present in unheated milk.

Riel and Sommer (1955) observed a correlation between oxidized flavor and the proteose-peptone N content in milk. Spontaneously oxidized milk contained a lower concentration of a proteose-peptone N and a lower heat stability than oxidation resistant milk.

Jenness (1957) isolated a proteose-peptone fraction--precipitated by salt but not by acid--which contained the principal constituent of proteose-peptone. This fraction was partially purified by fractional precipitation at varying values of pH and $(NH_4)_2SO_4$ concentrations. Later he (1959) suggested that this material corresponded to peak "5" and that it was the heat-labile loaf-volume depressant. Over 90% of this material had an electrophoretic mobility of -4.5×10^{-5} cm^2 $V^{-1}sec^{-1}$ at pH 8.6 (Veronal) and contained 1.2-1.3% P; soluble in the presence of $CaCl_2$ and not affected by rennin.

Aschaffenburg and Drewry (1959) observed that six bands were present in paper elcctropherograms of acid filtrates of heat-treated skimmilk. The major band corresponded to Larson and Rolleri's peak "5" and the five

small bands corresponded to peak "3." The same six bands were also found in concentrates from unheated milk, once again proving that the proteose-peptone are native constituents of milk and not artifacts formed by processing. These investigators found that the heterogeneous fraction could be separated from other whey proteins present in the casein-free filtrate at pH 4.6 by salting-out with sodium sulfate added to a concentration of 12 g/100 ml. They also observed that the proteose-peptone stained yellow with bromophenol blue on the filter paper strips, whereas other whey proteins formed normal bluish-green bands. Those yellow bands probably indicate that all bands contained carbohydrate.

The presence of carbohydrates in protein has been recognized for years. According to Gottschalk (1966), glycoproteins are defined as protein-carbohydrate complexes in which carbohydrates are covalently bonded to the poly-peptide chain. It cannot be dissociated without applying severe treatments such as milk acid hydrolysis. The carbohydrate moiety in proteins usually contains amino sugars such as glucosamine and galactosamine, sialic acid and hexoses such as galactose, mannose and fucose. The presence of glycoprotein in milk was first reported by Storch in 1897, who reported that the fat globule membrane contained carbohydrate. The presence of hexose

and hexosamine in the immune globulins (euglobulin and pseudoglobulin) was shown by Smith et al. (1946).

Nitschmann et al. (1957) reported that a glycomacropeptide derived from k-casein by the action of rennin contained a high level of carbohydrate. k-casein is the main glycoprotein of the micellar casein and was reported to contain about 5% carbohydrate (Alais and Jolles, 1961).

Thompson and Brunner (1959) determined hexose, hexosamine, fucose and sialic acid in several minor proteins of bovine milk, i.e., soluble membrane protein, Weinstein's minor protein and proteose-peptone. A high hexose and sialic acid content was a common characteristic of these fractions. They pointed out a similar relationship between glycoproteins of milk and blood. Further, they suggested that the glycoproteins of the proteose-peptone fraction probably could originate from blood serum.

Marier, Tessier and Rose (1963) indicated that 17 to 28% more sialic acid was present in the proteins precipitated with 12% trichloracetic acid than in casein precipitated at pH 4.5. Proteose-peptone contained 1.8% sialic acid which accounted for the entire difference between the sialic acid content of acid casein and the milk proteins precipitated with trichloracetic acid.

Ganguli, Gupta, Joshi and Bhalerao (1967) determined the sialic acid and hexose contents of the proteose-peptone and proteose fraction of milk. Proteose had

higher concentrations of sialic acid and hexose than the corresponding proteose-peptone sample. The casein fraction contributed the largest share of sialic acid to the sialic acid distribution in milk proteins. However the concentration of sialic acid in the proteose-peptone fractions was four times that found in the casein fraction. The sialic acid found in proteose and proteose-peptone was identified as a neuraminic acid derivative similar to that which appeared to be present in k-casein.

Brunner and Thompson (1961) reported chemical composition and physical parameters of five minor-protein fractions, namely: Rowland's proteose-peptone, Aschaffenburg's σ-proteose, Jenness' component 5, Weinstein's minor-protein fraction and the soluble fat globule membrane protein. The chemical composition of these five minor-protein fractions were very similar with respect to low contents of sulfur amino acids, contain carbohydrate, high in phosphorous and ash. Also some similarity was observed in electrophoretic anc ultracentrifugal properties. They concluded that a common component existed in these fractions. More recently McKenzie (1967) reviewed the status of minor phospho-glycoproteins in milk and suggested that the proteose-peptone should be considered as part of the casein complex.

Acid glycoproteins, as defined by Mehl et al. (1949), are carbohydrate-containing proteins which are

negatively charged at pH 4.5. Bovine serum acid glycoproteins, i.e., the M-1 component, are comparable to human orosomucoid and the M-2 group which were characterized by Weimer, Winzler (1955) and by Bezkorovainy (1962), respectively. Bezkoravainy (1965) isolated orosomucoid and M-2 glycoproteins from bovine serum and colostrum whey, and M-2 glycoprotein from milk whey. Milk whey contained no orosomucoid and only trace amount of the M-2 glycoprotein, which is less acidic than orosomucoid. Furthermore, both milk and colostrum contained a phosphoglycoprotein which had practically no absorption at 280 nm and behaved like the serum M-1 fraction in the isolation procedures. Physical and chemical properties corresponded to the major component of the milk proteose-peptone fraction. Therefore he concluded that close relationships could exist between colostrum glycoproteins and the proteose-peptone fraction.

The interrelationship between blood serum components and milk proteins was investigated by Hanson and Johansson (1959) by means of immuno-diffusion technique. They found that bovine milk contained twelve antigenic factors, six of which were related to bovine blood serum. Hanson (1959) assayed the bovine milk proteins which were related to blood serum by immune electrophoretic analysis of blood serum with its homologous immune serum absorbed with milk. Agar electrophoresis of the blood serum showed

seven fractions; prealbumin, albumin, α_1-, α_2-, β_1-, β_2- and γ-globulin. Immune electrophoretic analysis of the same serum with its antibody showed twenty-one precipitation lines. Only seven precipitation lines remained in the immunoelectropherograms after absorption of immune serum with bovine milk. Thus, fourteen proteins in bovine blood serum are similar to their counterparts in the milk protein system.

Recently Martinez-Resa, et al. (1969) identified bovine milk proteins by use of a similar technique. They observed twenty-six antigenic proteins in bovine milk by immunoelectrophoresis; six of these were of serum origin, seven were specific to whey, eleven were specific to casein, and two were of undetermined origin.

Ganguli, Agarwala and Bhalerao (1965) established a colorimetric method for the estimation of proteose-peptone in milk. 10% acetic acid and 16% trichloracetic acid were employed as precipitants. Recently, Joshi and Ganguli (1968) reported a turbidimetric method for the estimation of proteose-peptone, proteose and peptone in milk using 15% trichloracetic acid to precipitate the proteose-peptone and saturated ammonium sulfate to selectively salt-out the proteose fraction from the proteose-peptone fraction. Ganguli et al. (1965) reported that cow's milk contained 0.22% proteose-peptone which contained seventeen amino acids. Three components were observed by

paper electrophoresis at pH range 6.0 to 10.0. Cystine and serine were identified as the N-terminal amino acids. Bovine colostrum had a higher content of proteose-peptone than milk.

Ganguli's group (1966, 1967, 1968) made an extensive study of the effect of storage, trypsin action, rennet action and heat treatment on the proteose-peptone content of milk. Proteose-peptone N and non-protein N increased with storage at 30 C, presumably derived from other milk protein fractions such as casein and the whey proteins. The proteose-peptone content of milk and casein was increased by exposure to trypsin, supposedly by release from casein and eventually changing into non-protein nitrogen by further enzymatic degradation. The newly released "proteose-peptone" contained more components, possessing lower electrophoretic mobilities and lower sialic acid contents, than the native proteose-peptone content. The action of rennet on cow's milk nearly doubled its proteose-peptone content. They suggested the release of a proteose-peptone like material and non-protein fractions from casein which increased with increased rennet concentrations. Proteose-peptone-like material from casein and proteose-peptone from milk showed similarities in their electrophoretic patterns, sialic acid content and gel filtration patterns on Sephadex G-75. The levels of proteose-peptone in milk and ultracentrifugal whey were

measured to assess the effect of heat-treatment to 100 C for 15 min. A linear increase in proteose-peptone in the milk serum was observed accompanying gradual removal of micellar casein by ultracentrifugation. Neither of the principal whey proteins, i.e., α-lactalbumin or β-lactoglobulin, had an effect on the proteose-peptone level.

Shahani and Sommer (1951) observed an increase in the concentration of the proteose-peptone fraction when milk was pasteurized at 70 C for 30 min followed by homogenization.

Melachouris and Tuckey (1966) reported that the proteose-peptone fraction decreased from 0.1% to 0.06% by heating at 143 C for 2.08 seconds.

Kolar and Brunner (1965) found that component "8" present both in casein micelles and whey, was a tenacious contaminant of k-casein preparations and a leading zone in starch-urea gel electropherograms. Therefore, they suggested that it might be similar to λ-casein.

Recently Kolar and Brunner (1970) isolated components "5" and "8" from both heated and unheated skimmilk. Component 8 was fractionated into component 8-fast and 8-slow by gel-filtration chromatography on Bio-Gel P-10. Component 5 was characterized by its high proline and relatively low carbohydrate contents. Component 8-fast and 8-slow contained relatively high concentrations of phosphorus. All three components were void of cysteine

and cystine, and contained a low concentration of methionine.

Kolar and Brunner (1969) reported that component 5 and 8 existed in equilibria between micellar casein and the serum whereas component 3 was not present in the micellar casein. Similar results were observed by Ng, Brunner and Rhee (1970). They isolated lacteal serum component 3 from both heated and unheated skimmilk. Component 3 was characterized by its high content of carbohydrates and low content of aromatic and sulfur containing amino acids. Component 3 is referred to as the slow-moving component of the proteose-peptone fraction.

EXPERIMENTAL

Apparatus and Equipment

Raw milk was collected in five-gallon stainless steel cans and separated in a DeLaval disc-type separator or, for small scale preparation with a low-speed centrifuge (international Model V, Size 2). Either a Corning Model 12 pH meter or an Instrument Laboratory Inc. Model 245 Delta-Matic pH meter equipped with glass electrodes was used to minotor pH values. Celite filter aid on a Büchner funnel was used for clarification of the proteose-peptone-containing supernatant. Visking dialysis tubing from the Union Carbide Company was used for dialysis. For most laboratory weighings, a top-loading, direct-reading Mettler type K-7 balance was used. For analytical weighings, an Analytical Single Pan Balance manufactured by Sartorius was used.

Laboratory-constructed Plexiglass electrophoretic cells were used for acrylamide gel electrophoretic and immunoelectrophoretic analyses. A Heath direct current power source (voltage range of 0-400, and current 0-150 mA) was used for all electrophoretic runs. The excess dye was removed from the electrophoretic gels by means of an electrolytic destainer constructed of Plexiglass and equipped

with two stainless steel plate electrodes a 12-volt battery charger, operated at about 4 mA supplied the electric current.

Protein solutions were freeze-dried on a laboratory-constructed lyophilizer. Lyophilized protein samples were dried to equilibrium in a vacuum desiccator over P_2O_5 at approximately 25 C.

Gel filtration chromatography (desalting samples) was accomplished in a glass column (1.9 x 4.5 cm) packed with Bio-Gel P-2 beads. The eluates from the columns were monitored at 254 nm with a recording ultraviolet analyzer Model UA-2 manufactured by Instrumentation Specialities Company.

When glycoprotein-staining was performed, acrylamide gel slabs were shaken in a New Brunswick Scientific Company Model No. RW-150 shaker. A micro-Kjeldahl apparatus was used for nitrogen determination. A Beckman Dk-2A spectrophotometer equipped with silica cells (1 cm path length) was used for all spectrophotometric measurements.

Immuno-doubleddiffusion by the Ouchterlony technique was performed in Petri-dishes and immunoelectrophoresis was done on 5 x 10 cm glass plates or microscope slides. A hand magnifying glass was used for making a visual examination of the precipitation lines.

A Polaroid Land Camera (Model MP-3) was used to photograph the electropherograms, immunodiffusion plates and immunoelectropherograms.

Chemicals and Materials

The milk used for this study was obtained from Holstein cows constituting the Michigan State University dairy herd. All milk specimens were collected at the time of milking and immediately separated.

Cow blood was collected, allowed to clot and the serum recovered by centrifugation. Rabbit anti-sera against proteose-peptone and bovine blood serum were prepared locally.

The principal chemicals used in this research and the suppliers are given below. Tri-hydroxymethyl amino-methane (Sigma 7-9) was used to prepare buffers. Reagent grade ammonium persulfate was purchased from Baker Chemical Company. Phenol was acquired from Mallinckodt and redistilled prior to use. The N,N,N',N'-tetra-methyl-ethylenediamine were obtained from Eastman Organic Chemicals.

Cyanogum 41 used in acrylamide gel electrophoresis was purchased from E. C. Apparatus Company. Bio-Gel P-2 was obtained from the Bio-Rad Corporation and a 1% solution of dimethyldichlorosilane in benzene was obtained from Pfanstiehl Laboratories. Mannose was obtained from Fisher Scientific Company. Tryptophan was acquired from

Calbiochem. Basic fuchsin was purchased from Allied Chemical Corporation.

Diethylbarbituric acid and sodium diethyl barbituric acid for making the veronal buffer were purchased from the Fisher Scientific Company. Glycine and the boric acid used for buffers were obtained from Fisher Scientific Company.

Agar used for immunological assay was obtained from Difco Laboratory and dialyzed before use.

Preparation of Protein Samples

Proteose-peptone

Uncooled, raw milk collected in a stainless steel container was defatted by separation or low-speed centrifugation at 1,000 g for 20 min. Skimmilk was heated to 95 C for 20 min in a Pyrex Erlenmeyer flask placed in a boiling water bath, then cooled to 25 C with running tap water. The pH was adjusted to 4.6 with either glacial acetic acid or 1N-HCl. After standing 30 min, the precipitated casein and heat-denatured whey proteins were filtered off with a Büchner funnel. A Whatman No. 42 filter paper covered with Celite filter aid served as the filtration media. The clear, grennish filtrate was concentrated with a vacuum concentrator or by pervaporation. When concentrated approximately fourfold, the clear greenish filtrate appeared slightly mucoidal. Eventually,

a small amount of white precipitate developed. The concentrated solution was divided into two fractions. One fraction was desalted by dialysis and the other was desalted on a Bio-Gel P-2 column. Details of the desalting procedure are described in the section on sample purification.

The isolation procedure for the proteose-peptone fractions is presented diagrammatically in Figure 1.

Whole Bovine Blood Serum and Heat-stable Bovine Serum

Whole bovine blood was allowed to clot by standing at room temperature for a period of one to two hours and, thereafter at 4 C overnight. Then, the serum exudate was centrifuged and the clear supernatant layer removed. A fraction of the supernatant was dialyzed and lyophilized for a subsequent preparation of desalted, whole bovine serum.

The preparation of heat-stable bovine serum was accomplished by a two step heating procedure. First, the bovine serum was heated to 80 C for 20 min to coagulate the bulk of the albumin and other heat-coagulable proteins. Then, the clarified serum was again heated to 90 C for 20 min and filtered. The filtrate was dialyzed against three to four changes of deionized water for three days at 4 C. The dialyzed sample was pervaporated and lyophilized. This preparation procedure is outlined in Figure 2.

Purification of Samples

Proteose-peptone was desalted either by dialysis or by passing through a P-2 column. The greenish, clear supernatant, containing proteose-peptone, was adjusted to pH 3.0 and dialyzed at 4 C against three or four changes of deionized water previously adjusted to pH 3.0 with acetic acid. Next, the pH was adjusted to 7.0 and dialyzed at 4 C against three or four changes of deionized water. Dialysis time was from twelve to twenty-four hours for each change of water.

Blood serum and heated serum filtrate were also dialyzed against three or four changes of deionized water.

A fraction of the proteose-peptone was desalted over a Bio-Gel P-2 column. Once lyophilized, the protein preparation was difficult to resolubilize; a 2% concentration being its maximum solubility. It was observed that when the proteose-peptone was carried in acidic solution it was more soluble, therefore the protein was maintained in an acidic environment during the desalting step (ammonium acetate at pH 4.6) over the column.

The Bio-Gel P-2 beads were hydrated overnight by soaking in deionized water followed by equilibration with an ammonium-acetate buffer at pH 4.6. Glass chromatographic columns with inside dimensions of 1.9 cm in diameter and approximately 45 cm in length were coated with dimethyldichlorosilane to prevent buffer from flowing faster at the

glass-bed interface. Thus, a 1% solution of dimethyldichlorosilane in benzene, warmed to approximately 60 C was poured into a clean column. After pouring off the solution, the residual benzene was evaporated in a drying oven. The chromatographic column was filled with buffer and a small amount of glasswool placed at the bottom of the column. A wide-stem funnel was attached on top of the column and the equilibrated beads were poured in with constant stirring. Following the formation of a small layer of beads at the bottom of the column, the outlet was opened to allow the bed to pack more tightly and to complete the packing. The final column height was 31.5 cm. The void colume (V_o) was determined by eluating a solution of Blue Dextran 2,000 through the column. A disc of filter paper was placed on top of the gel to prevent its disturbance when the sample was applied. The 4- to 8-fold concentrated proteose-peptone solution was applied to the column in approximately 10 to 15 ml portions with a 20 ml syringe.

The column effluent was monitored at 254 nm. The fraction representing the first peak on the recorder was collected and lyophilized. The lyophilized samples were further dried in a vacuum desiccator at room temperature over P_2O_5 for two to three days in preparation for chemical analysis.

Composition Analysis of Samples

Nitrogen

A micro-Kjeldahl apparatus, employing round bottom digestion flasks and a steam entrainment arrangement was used for the nitrogen determinations. Duplicate samples of approximately 15 mg of dried protein were digested with 4 ml of the digestion mixture over a gas flame for approximately 1 hr. The digestion mixture consisted of 5.0 g $CuSO_4 \cdot 5H_2O$ and 5.0 g SeO_2 in 500 ml of concentrated sulfuric acid. After cooling the digestion mixture, 1 ml of 30% H_2O_2 was added to each flask and digestion continued for another hour. The digestion flasks were cooled and the sides were rinsed with 10 ml of deionized water. Then, the digestion mixtures were neutralized with approximately 25 ml of 40% sodium hydroxide solution. The digestion flask was connected to the distillation apparatus and the released ammonia was steam distilled into 15 ml of a 4% boric acid solution containing four to five drops of indicator. The indicator consisted of 400 ml of bromocresol green and 40 mg of methyl red in 100 ml of 95% ethanol. Distillation was continued until a final volume of 65 ml of the distillate was collected. The ammonia-borate complex was titrated with 0.0198 N HCl. Tris-hydroximethyl aminomethane (Sigma 121) was used as the primary standard to determine the normality of the hydrochloric acid. The titrations were performed manually. The average

recoveries of a tryptophan standard performed in triplicate was 98.30%.

Carbohydrates

The Molisch test served as a qualitative estimate for the presence of carbohydrates in the samples. Two drops of a naphtol solution was added a small volume of a 1% solution of protein; glucose was employed as a color standard. After the addition of 1 ml of concentrated H_2SO_4 down the side of the tube, the development of a purple color at the acid/solution interface was noted. The proteose-peptone solution showed a red-violet color similar to that obtained with a glucose standard. However, heated bovine serum was a brownish-purple, indicating the presence of other chromogenic materials.

Protein-bound hexose analyses were performed colorimetrically by the method of Dubois et al. (1956). Two to four milligrams of proteose-peptone and 3.5 to 6 mg of heated bovine serum were placed in separate test tubes. Three milliliters of deionized water and an equal volume of 5% (v/v) phenol solution were added and mixed. Reagent grade phenol was redistilled for preparation of the 5% solution. Then, 15 ml of concentrated sulfuric acid was added rapidly and agitated vigorously for 30 sec. Constant mixing of the reaction mixture was critical to the reproducibility of the assay. After 10 min of standing, the tubes were stirred again and placed in a

water bath at 25 to 30 C for 30 min. The percentage transmittance was read at 490 nm with a Beckman DK-2A spectrophotometer. The standard curve (Figure 3) was prepared for each assay from a 1:1 (w/w) mixture of galactose-mannose.

Characterization of the Samples

Polyacrylamide Gel Electrophoresis in a Discontinuous Buffer System

The procedure for preparing and the performing discontinuous acrylamide gel electrophoresis was adapted from the method described by Melachouris (1969). The gel system was prepared by using two working solutions, consisting of a running gel and a spacer gel. The running gel solution was prepared by dissolving 90 g of Cyanogum 41 in 0.380 M tris-HCl buffer, pH 8.9 and making the volume to 1,000 ml. For the spacer gel, 5% (w/v) Cyanogum 41 was prepared in 0.062 M tris-HCl buffer, pH 6.7. The working gel solutions were filtered and stored at 2 to 3 C. All solutions were brought to room temperature before use. When the gel bed was prepared, N,N,N',N'-tetramethylethylenediamine and freshly prepared 10% (w/v) ammonium persulfate solution were added in a ratio of 0.1 ml and 1 ml per 100 ml of gel, respectively, while vigorously stirring. The gel bed was constructed of Plexiglass and was equipped with a small chamber underneath the bed surface for the circulation of cooling water (see Figure 4).

Before the gel solutions were poured in the gel bed, a Plexiglass divider was placed 15 cm from one end of the gel bed. Silicone grease was employed to prevent seepage of the gel-forming solution into the void area. The buffer/gel connecting holes in the bed legs were sealed with electrical insulation tape. The larger area of the gel bed was filled with 190 ml of running gel. After polymerization under nitrogen, the divider was removed. Then, the smaller area of the bed was filled with 90 ml of the spacer gel solution. A slot former (1x0.1x0.3cm) was inserted into this gel solution at a distance of 0.5 cm behind the running gel.

The protein samples were dissolved in spacer gel solution, diluted 1:1 with deionized water, at approximately 4% protein concentration. Bromophenol Blue was added to one sample slot as a marker. Approximately 30 to 40 µl samples were applied to the slots with a microsyringe and the slots covered with a Plexiglass bar. The gel was covered with Saran Wrap to reduce evaporation, then connected to electrode vessels containing approximately 1800 ml of 0.046 M tris-glycine buffer. Electrophoresis was carried out at 15 C at a current of 80 to 85 mA in a range of constant voltage of 180 to 200 volts. When the dye marker migrated 12 to 13 cm from the sample slots, usually 10 to 12 hr, electrophoresis was completed. The gel was removed from its frame and stained for 5 to

10 min in an amido black solution consisting of 250 ml water, 250 ml methanol, 50 ml of glacial acetic acid and 5 g of amido black 10B (Naphtol blue black). The unbound dye was removed electrophoretically in an electrophoretic destainer cell containing 7% acetic acid. Electropherograms were photographed with a Polaroid MP-3 Land Camera.

Polyacrylamide Gel Electrophoresis in a Continuous Buffer System

The procedure followed for acrylamide gel electrophoresis in a continuous buffer system was essentially similar to that described above with the exception that a single buffer system was employed and no spacer gel was poured.

The buffer was prepared by diluting a stock borate buffer 1:2.5 with deionized water. The stock borate buffer was prepared by dissolving 881 g boric acid and 190 g sodium hydroxide in 19 liters of water, adjusted to pH 8.6. The gel solution was prepared by dissolving 80 g of Cyanogum 41 in 1 liter of borate buffer and filtering. To 250 ml of this solution, 0.3 ml of N,N,N'N'-tetramethylethylenediamine and 3 ml of freshly prepared 10% (W/V) ammonium-persulfate were added. Within 30 sec, the solution was poured into the gel bed. The slot former was positioned and the gelling mixture was polymerized under an atmosphere of nitrogen.

The gel bed was connected to the electrode vessels by gel-filled legs which rested in tanks containing about

1,800 ml of borate buffer prepared as described above. After the slot former was removed, 30 to 50 µl of 4 to 5% protein solution was applied into each slot. The gel was covered with Saran Wrap and platinum electrodes were inserted into the buffer tanks. Electrophoresis was performed for approximately 8 to 10 hr with a current 80 to 85 mA and voltage range of 180 to 200. A drop of bromophenol blue solution was introduced into one slot as a marker since bromophenol blue usually migrates with the fastest moving proteins. After the completion of electrophoresis, the gel was stained in the same way as previously described.

Staining for Glycoproteins in the Proteose-peptone and Heated Bovine Serum in Acrylamide Gels

Carbohydrate-containing proteins of proteose-peptone and heated bovine serum were visualized by several available methods. The technique described by Zacharius et al. (1969) was most satisfactory in this study. Following electrophoresis, acrylamide gels were washed thoroughly with distilled water to remove the buffer ions before beginning the staining procedure. The washed gel was cut in half. One part was stained with amido black and the other was developed for glycoproteins. The latter method was performed as follows: immerse the gel in 12.5% (w/v) trichloroacetic acid for 30 min to fix the protein. Then,

the lightly rinsed gel was treated with 1% periodic acid in 3% acetic acid. The gel was washed six times for 10 min each or overnight with a few changes of water to remove excess periodic acid. Complete removal of the periodic acid was imperative to obtain a stained gel with clear background. Then, the gel was exposed to a fuchsin-sulfite solution in the dark for 50 min. The fuchsin-sulfite stain was prepared as follows: 16 g of potassium meta-bisulfite was dissolved in 2 liters of water and exactly 21 ml of concentrated hydrochloric acid were added. Eight grams of finely powdered basic fuchsin was added and the mixture stirred gently with a mechanical stirrer for 2 hr at room temperature. After standing for 2 hr the solution was passed through a column packed with alternate layers of activated charcoal and decolorizing carbon. The column effluent was colorless and was stored in the cold room.

The stained gel was washed with freshly prepared 0.5% meta-bisulfite solution three times for 10 min each and then washed with frequent changes of distilled water until excess stain was removed. The gel was stored in 3 to 7.5% acetic acid and photographed for a permanent record. Table 1 shows the steps in the staining procedure.

Immunological Analysis

Preparation of rabbit anti-sera against proteose-peptone and bovine serum.--Immune-sera against

TABLE 1.--Staining of glycoproteins in acrylamide gel

Step	Gel Treatment	Time Interval (min)
1.	Wash gel in shaker with a few changes of water.	60
2.	Immerse in 12.5% trichloracetic acid.	30
3.	Rinse lightly with distilled water.	0.25
4.	Immerse in 1% periodic acid (made up in 3% acetic acid)	50
5.	Wash 6 times for 10 min in distilled water or wash overnight with a few changes of distilled water.	60 or ON
6.	Immerse in fuchsin-sulfite[a] stain in a dark room.	50
7.	Wash 3x for 10 min with freshly prepared 0.5% meta-bisulfite solution	30
8.	Wash with frequent changes of distilled water until excess stain was removed.	ON[b]
9.	Store in 3 to 7.5% acetic acid. (Note: 200 ml of reagents was used for each step.)	

[a]Prepared according to McGuckin and Mckenzie (1958)

[b]Overnight

proteose-peptone and bovine blood serum were obtained from hyperimmunized rabbits. The antigenic proteins were dialyzed proteose-peptone (12.22% N) and dialyzed bovine blood serum (13.21% N).

Fifty milligrams of each antigen were dissolved in 1 ml of Jenness and Koop's buffer to which was added 1 ml of Freund's complete adjuvant. After the second injection, Freund's incomplete adjuvant was employed. A total of six injections were made during a two month period. The route of injection was alternately deep subcutaneous and intramuscular at intervals of two weeks. Injections were made at weekly intervals until the fourth injection. A small amount of serum was collected and checked against the antigen. Because of the low apparent titer, booster shots of antigen were administered. The injection intervals, route of injection and kind of adjuvant used are presented in Table 2.

Immuno-doublediffusion.--The technique for double diffusion reactions in petri dishes was adapted from Ouchterlony (1949). On occasion, a micro-doublediffusion technique developed by Wadsworth (1957) was employed. The size and distance of the antigen and antibody well are diagrammed in Figure 5. In most experiments the antigen and antibody wells were formed by using LKB template 6808A (template pattern A in Figure 5). Some patterns were formed manually by using a cork borer.

TABLE 2.--Timetable for immunization procedure

Injection No.	Interval	Route of Injection	Adjuvant	Total volume
1.	1st day	Intramuscular	Freund's complete	2 ml containing 50 mg protein
2.	6th day	"	"	"
3.	13th day	Subcutaneous	Freund's incomplete	"
4.	20th day	"	"	"
	37th day	Testing for antibody titer with small amount of blood		
5.	48th day	Intramuscular	Freund's complete	2 ml containing 50 mg protein
6.	55th day	Subcutaneous	"	"
	65th day	Collected whole blood		

A 2% purified Difco agar, containing 0.85% NaCl, was employed as the diffusion media. This was prepared as follows: 500 g of a previously dialyzed 4% agar was melted, 8.5 g of NaCl was added and the volume made up to 1 liter. Enough sodium azide was added to yield a final concentration of 0.02%. This solution was divided into 200 ml fractions and stored in the cold room.

All the plates and slides were boiled in weak detergent solution for 3 min, rinsed several times in distilled water, placed in 95% ethanol for 3 min and wiped dry with Kimwipes. Templates were also washed (but not boiled) and coated with a thin layer of silicone vacuum grease. Before pouring the melted 2% agar, the surface of plates and slides were precoated with a 0.2% agar solution. The excess agar was removed by tilting and allowed to dry in a low temperature oven (60 C to 70 C) for 20 min.

Ten to twelve milliliters of the agar solution were poured into the petri dishes and 2.2 to 2.5 ml were pipetted onto each microslide. The agar was allowed to solidify for 20 min in a humid chamber. Antigen and antibody wells were punched using a suitable pattern or template. The agar plugs in the small wells were removed by means of a Pasteur pipette connected to a water suction pump.

Antigenic proteins dissolved in Jenness and Koop's buffer and anti-sera were introduced into appropriate

wells with a micro-syringe, being careful to avoid entrapped air bubbles. For most experiments, the antigen concentration was 2%. Heated bovine serum was quite insoluble thus its solution appeared as a cloudy suspension.

Controls for the absorption experiments were prepared by diluting anti-serum with an equal amount of homologous or heterologous antigen. The reactant-charged plates and slides were placed in moistened chambers for two to five days. In some experiments, the antigen and antibody wells were refilled on the second day.

In the larger plate tests, precipitation bands were recorded through three to five days. The reactions in the microscope slides were fully developed within three days. Examination of the precipitation lines was performed by means of magnifying glass (5x), holding the glass slide in front of a dark background with cross lighting. For the recording of immunoprecipitates, plates and slides were stained and photographed. This was accomplished as follows: the plates and slides (gels) were soaked in a 0.15 M NaCl for 24 to 48 hr. No precipitation lines disappeared as a result of this treatment. In fact, the precipitation lines became more prominent. To enhance visualization of the reaction zones, either a thiazine red (0.1% in 1.0% acetic acid) or an amido black staining solution (1 g of amido black 2B, 450 ml of 1 M acetic acid, 450 ml of 0.1 M sodium acetate and 100 ml of

glycerol) was employed. The amido black stain was more satisfactory with slides and both stains were extremely difficult to remove from the thicker gels on the Ouchterlony plates. The destaining operation consisted of successive changes of 2% acetic acid solution until the background color was removed.

Immunoelectrophoresis.--Immunoelectrophoresis is a combination of conventional agar gel electrophoresis and an immunological reaction in gel. The original immunoelectrophoresis method developed by Grabar (1953) was performed as illustrated in patterns J and K in Figure 5. A micro-immunoelectrophoresis method as modified by Scheidegger (1955) was performed on microscope slides using patterns H and I, Figure 5.

Purified agar was used for the gel matrix. The preparation of a 1% agar in veronal buffer pH 8.6, ionic strength 0.05 was as follows: dialyzed agar (500 g of 4% gel) was melted in a water bath and made to 1 liter with deionized water. Then, the agar solution was diluted 1:1 with veronal buffer which was prepared by dissolving 1.26 g barbital, 3.8 g of sodium acetate and 9.74 g sodium barbital in 2 liters of deionized water. Agar reaction gels were prepared as previously described for immuno-diffusion technique.

A series of six microslides or two macroslides at a time were positioned in the electrophoresis chamber (see Figure 6) containing approximately 1 liter of veronal buffer, equally divided between the cathodic and anodic chambers. Electrophoresis was conducted at 4 mA per microslide at room temperature. Each macroslide was considered as two microscope slides (i.e., 8 mA per slide). The electrical contact between the agar gel and the electrode vessels was accomplished by means of strips of filter paper wetted in the buffer solution. A small amount of bromophenol blue was placed in one of the antigen wells nearest the cathode and the current was applied until the dye had migrated 2.5 to 3.0 cm in the microslides and 3.5 to 4.0 cm in the macroslides. Approximately 30 min and 60 min, respectively, were required. Following electrophoresis, the anti-serum channels were filled with appropriate immunesera, Precipitation bands were characterized after 24 to 72 hr of diffusion. After 24 hr the anti-sera troughs were refilled with antibody. Precipitation zones were recorded at 48 hr for the microslides and at 72 hr for the macroslides. The staining procedure was similar to that used for the immunodiffusion slides. The stained slides were wrapped with filter paper to prevent gel peeling and air dried in preparation for photographic recording.

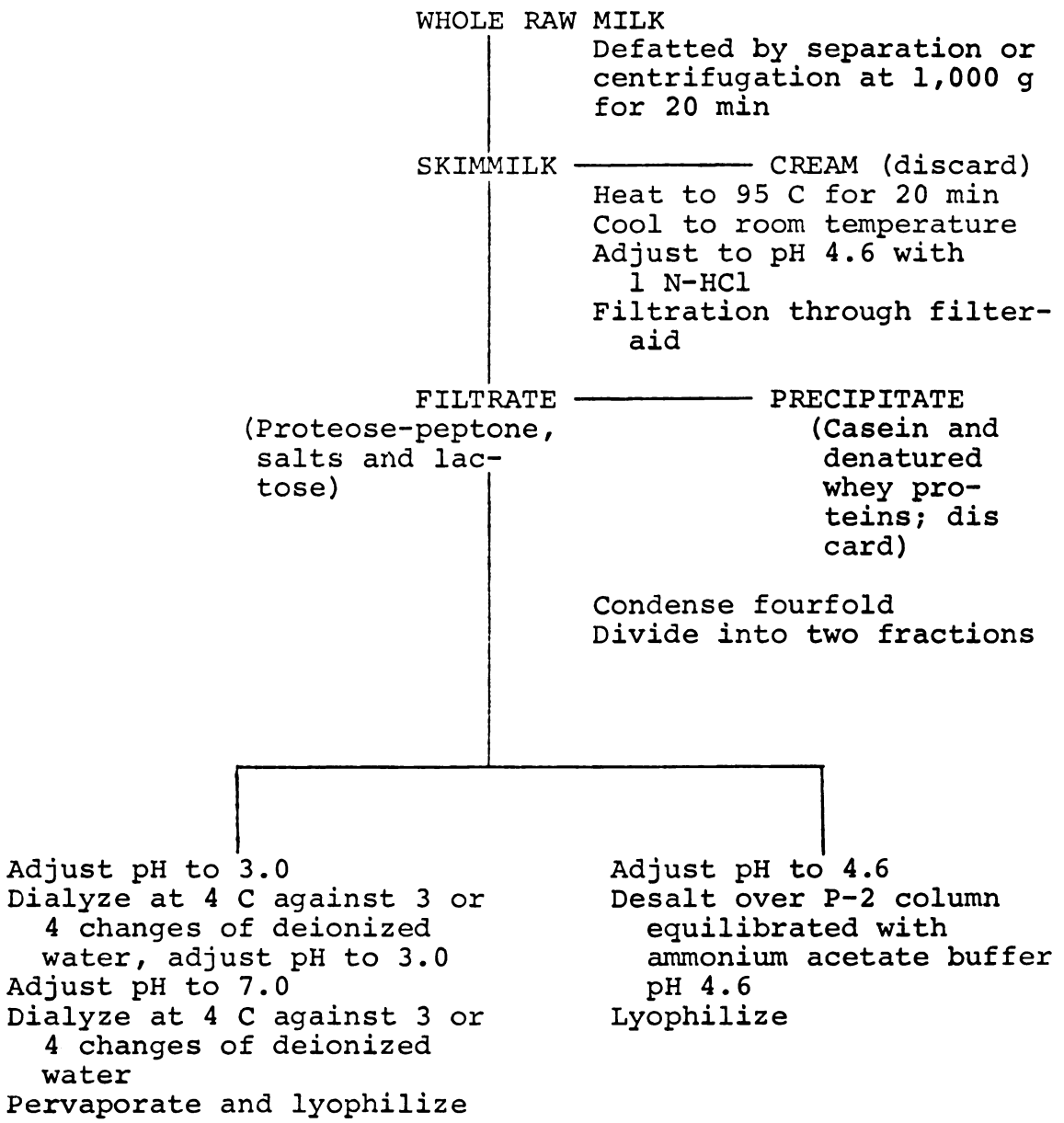


Figure 1.--Procedure for the preparation of proteose-peptone.

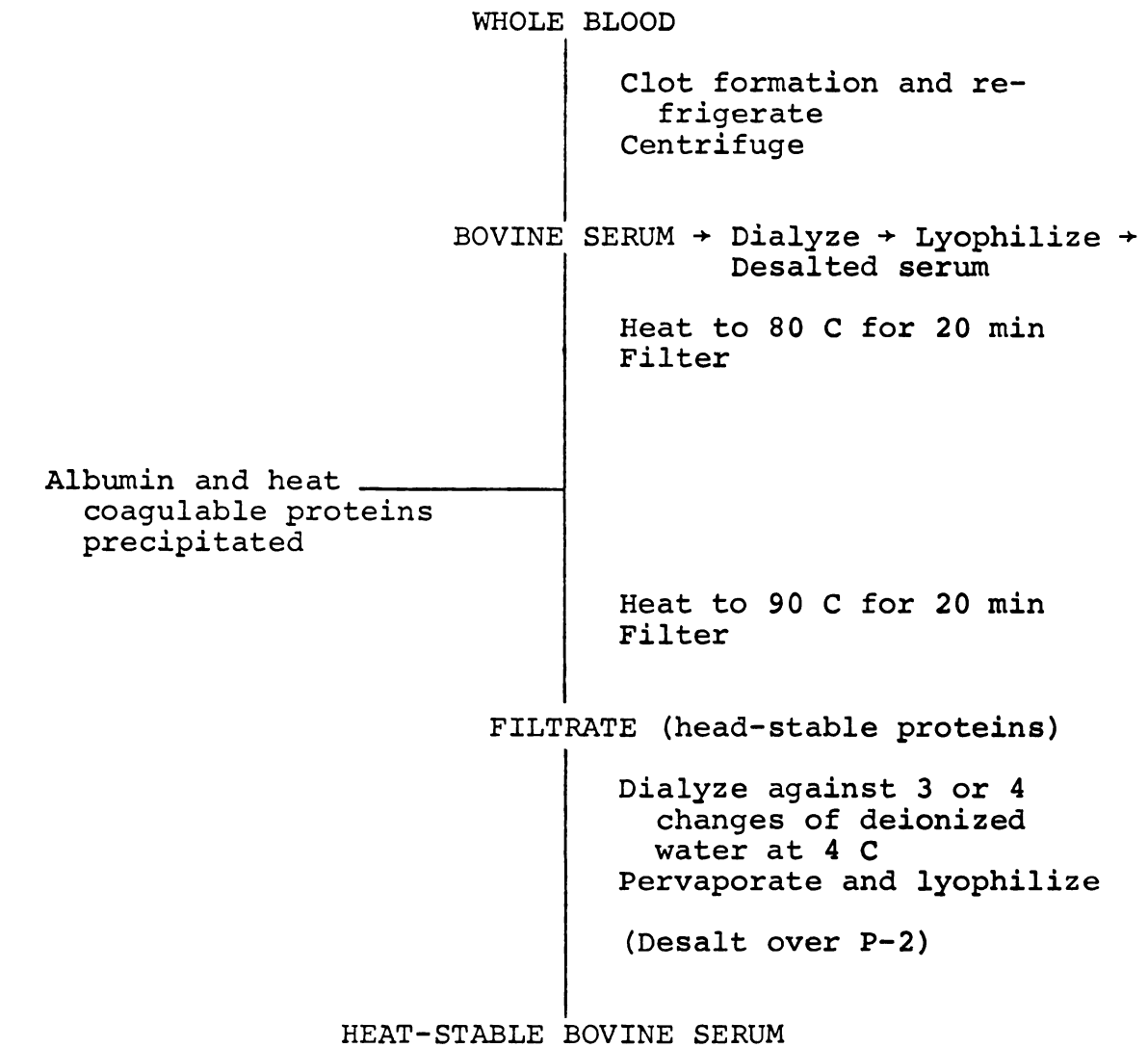


Figure 2.--Procedure for the preparation of bovine serum and heat-stable bovine serum.

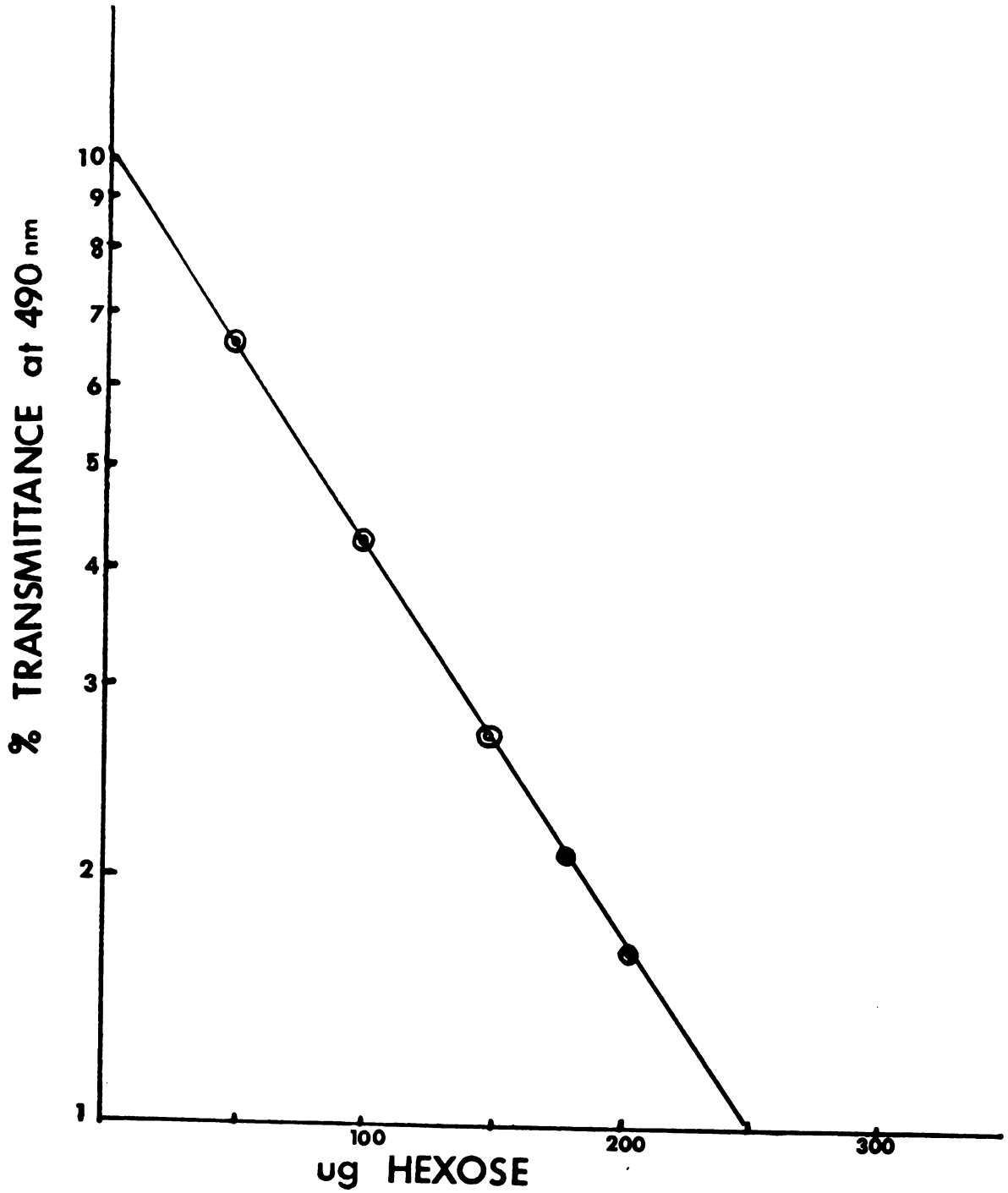


Figure 3.--Standard calibration curve for the spectrophotometric determination of hexose; 1:1 (w/w) mixture of galactose and mannose.

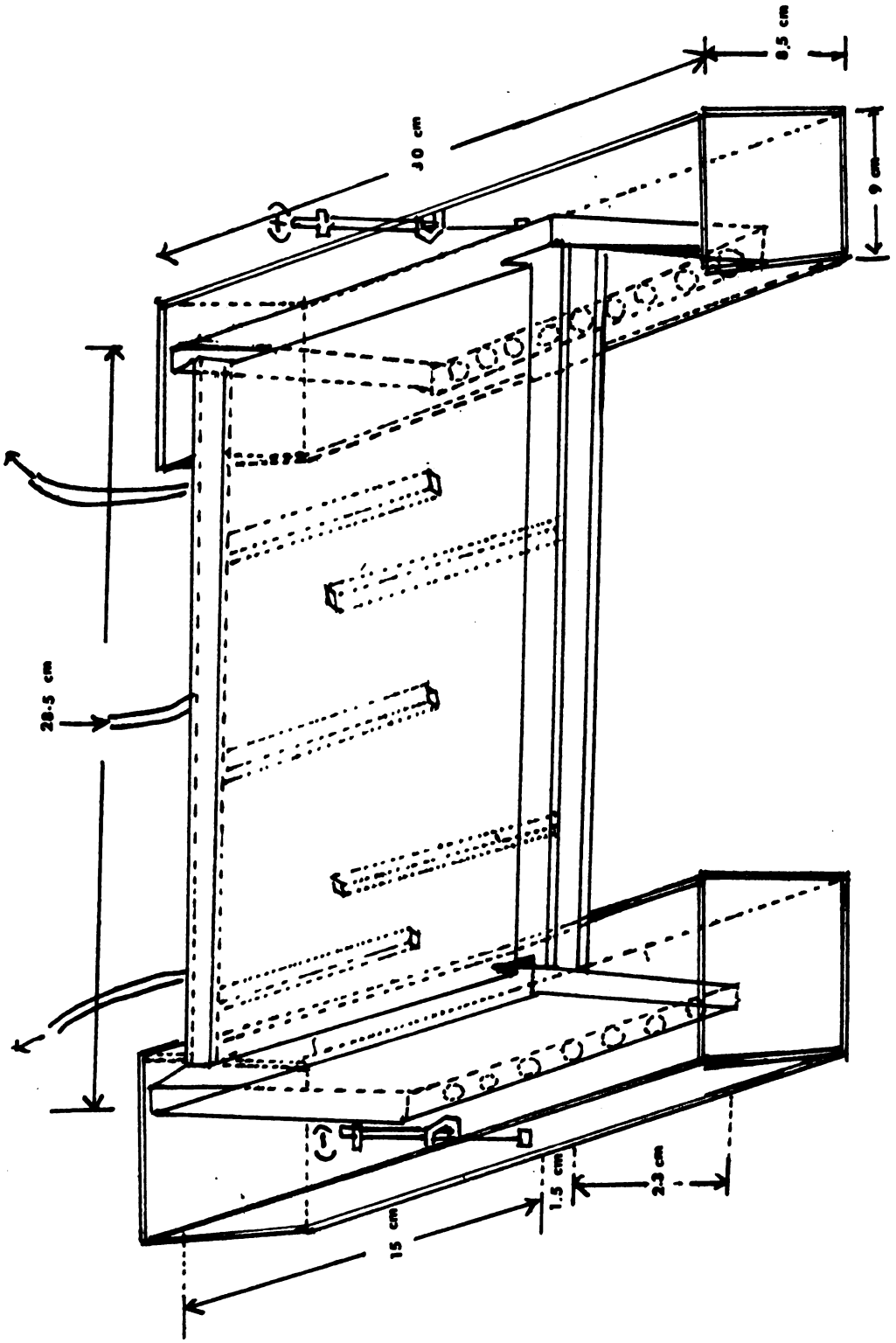


Figure 4.--A diagrammatic representation of the horizontal gel-electrophoresis bed (Plexiglass) used in this study.

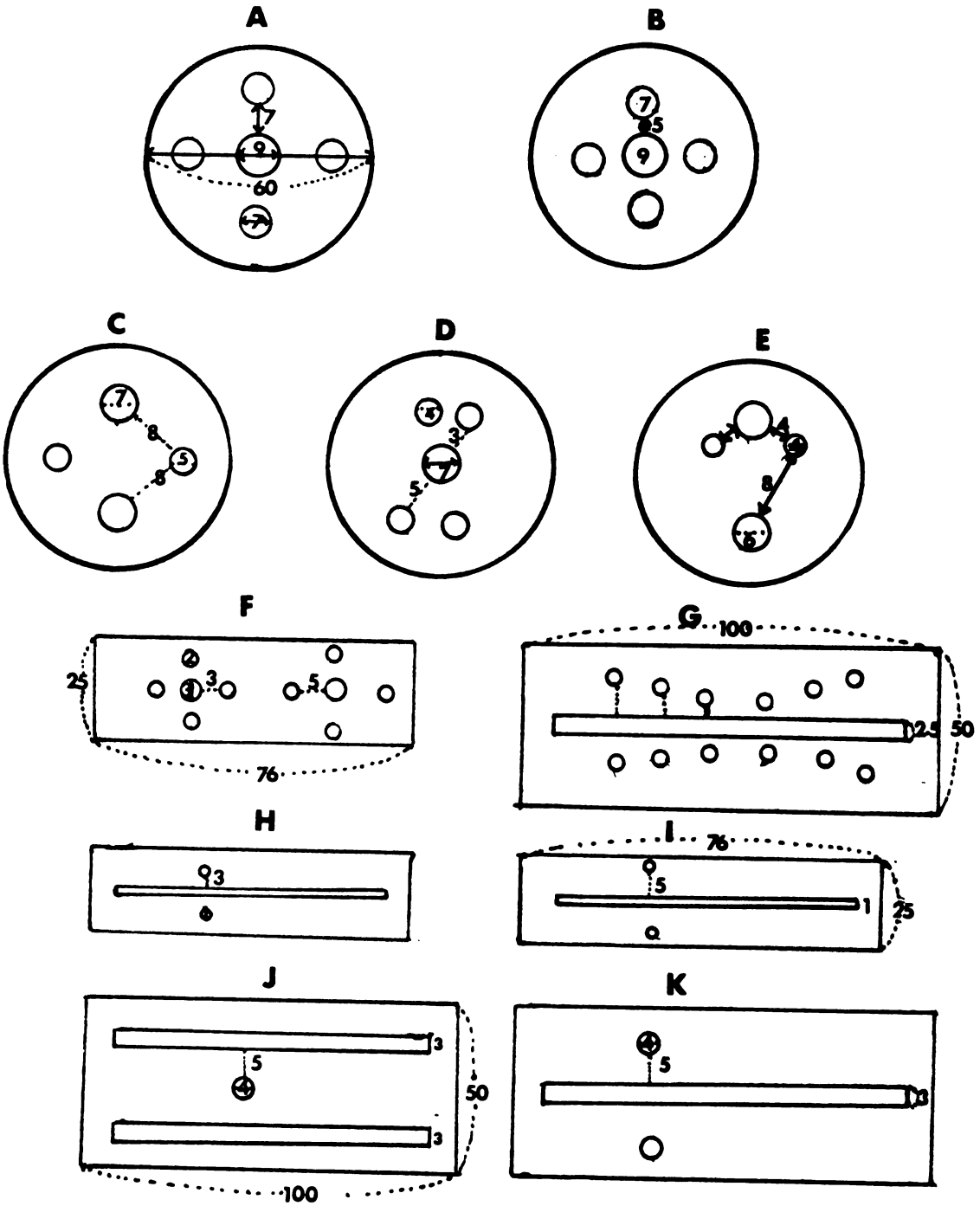


Figure 5.--Template patterns used for the immunodoublediffusion and immunoelectrophoresis experiments. Size and distance between antigen and antibody wells are indicated in mm units.

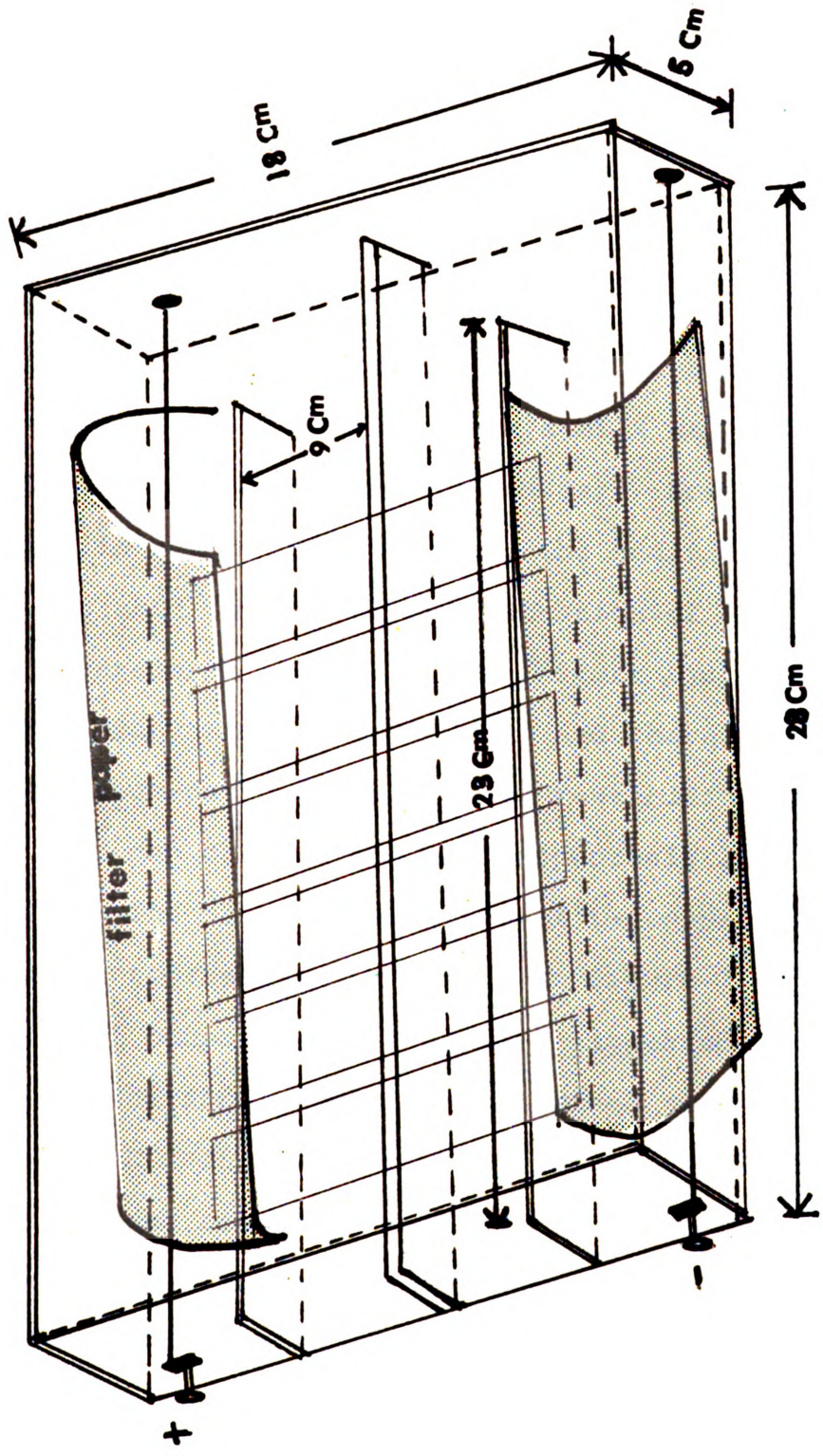


Figure 6.--A diagrammatic representation of the electrophoresis chamber (Plexiglass) used in this study for the immunoelectrophoresis experiments.

RESULTS AND DISCUSSION

Preparation of Proteose-peptone, Bovine Serum and Heated Bovine Serum

The isolation procedure used to prepare the proteose-peptone fraction from cow's milk was adapted from the method described by Rowland (1938a). However, the final step in his procedure--i.e., the addition of trichloracetic acid to a final concentration of 8%--was eliminated. Therefore, the final supernatant includes all non-protein substances. The purity and characterization of the proteose-peptone fraction were established by analysis for nitrogen content and electrophoresis in acrylamide gels. The electropherograms of two separate preparations are represented in slot 2 in Figure 7, A and B. The principal components--3, 5 and 8--appeared in both patterns. Additional minor protein zones were apparent in slot 2 of B. This slightly different gel electrophoretic pattern seemed to reflect minor experimental variations in the preparative procedures.

Preparation of bovine serum and heat-stable serum was outlined in Figure 2. The proteose-peptone fraction in whey is analogous to the heat-stable serum fraction in serum. Logically, in order to study the interrelation between proteose-peptone component and bovine blood serum,

one should isolate the counterpart of the proteose-peptone from bovine serum in a similar manner. Not many studies have shown the effect of heat-denaturation on the bovine serum components. Tekman and Öner (1966) demonstrated that blood serum samples heated for 15 min at 65 C showed a decrease in the electrophoretic mobility of albumins and an absence of globulins. Samples heated to 75 C showed no migration of any protein fraction by paper electrophoresis. Studying the heat denaturation of serum proteins in milk, Larson and Rolleri (1955) found that the serum albumin in milk, which is identical with serum albumin of blood (by Coulson, 1950), was heat denatured completely by heating to 75 C for 30 min. An electrophoretic distribution study of serum protein showed that it contained 41.0% albumins, 13.0% α-globulins, 8.2% β-globulins and 37.8% γ-globulins (Svensson, 1946). The heat treatment in the present study was accomplished in two steps of two different temperatures: (80 C) and (90 C) for 20 min, respectively.

According to the above mentioned workers, all the serum albumin in serum seemed to be heat denatured. Acrylamide gel electropherograms heated bovine serum (slot 3 in Figure 8A, B) showed an absence of the albumin zone. The three minor bands observed for heated bovine serum seemed to be obscured by the high content of heat-labile protein in normal bovine serum. The possibility

exists that the zones observed in the heated serum are artifacts resulting from the heat treatment. Tybor et al. (1970) reported that approximately 30% of the bovine serum proteins remained after heating to 80 C, which is somewhat divergent from the results of other workers.

Purification of Samples

The proteose-peptone-containing filtrate includes high contents of lactose and salts. When this crude proteose-peptone fraction was applied to electrophoretic gels, the resulting zones were diffuse and poorly resolved. Thus, the proteose-peptone fraction was desalted by either dialysis or passing through a Bio-Gel P-2 column. Dialysis was first performed at a low pH (~3.0) in order to remove calcium and phosphate. These are withdrawn from the colloidal particles as the pH is decreased by adding acid (Kolar and Brunner, 1970). The purity was assessed by determining the nitrogen content. As shown in Table 3, there was a very low nitrogen content in the 48 hr dialyzed material, which was attributed to the high salt contents. Additional dialysis removed most of the non-protein components.

Electropherograms of dialyzed proteose-peptone are presented in slot 2 of plates A and B, Figure 7. Slots 1 in A and B show electrophoretic patterns of proteose-peptone preparations desalted over the P-2

TABLE 3.--Nitrogen content of proteose-peptone, heat-stable bovine serum and bovine serum; and comparison of proteose-peptone nitrogen content between this study and other workers

Component / Desalting method	Nitrogen (%)			
	Proteose-peptone		Heat-stable bovine serum	Bovine serum
	Preparation I	Preparation II		
Dialysis 48 hr	-	4.01	7.24	-
Dialysis 72 hr	12.22	-	-	-
Dialysis 96 hr	-	12.08	8.81	13.21
Bio-Gel P-2	13.68	11.98	-	-
Brunner[a] (Proteose-peptone)	13.70-13.90			
Ng[b] (component 3)	13.1			
Kolar[c] (component 5)	13.8			
Kolar (component 8)	13.9			
Bezkorovainy[d] (phospho-glycoprotein)	13.6			

[a]Brunner and Thompson (1961)

[b]Ng and Brunner (1967)

[c]Kolar and Brunner (1968)

[d]Bezkorovainy (1965)

column. In both preparations, component 3 of the proteose-peptone fraction was lost by passing through the P-2 column. The only possible explanation is that component 3, which exhibits the largest molecular weight among the proteose-peptone components, was excluded by the P-2 column before sample collection began. The difference between preparations shown in A and B, Figure 7 was that the former was concentrated fourfold and the latter eightfold. The clear greenish filtrate containing proteose-peptone became cloudy and a white precipitate formed when it was concentrated more than fourfold. The white precipitate was suspected as being aggregated component 3. However, upon gel electrophoresis it showed a characteristic proteose-peptone pattern.

The effluent from the column was minotored at 254 nm. The fraction corresponding to the first peak was collected. As shown in Figure 9, the elution profile of proteose-peptone did not return to a baseline position between the proteose-peptone peak and the second large peak, consisting of lactose, salts and other soluble non-protein components of whey. Therefore the proteose-peptone collected contained small amounts of these components.

Composition Analysis of Samples

Total nitrogen content was determined by a micro-Kjedahl method. The determination was done in triplicate

and the accuracy of the determination was checked by recovery of nitrogen from known amounts of tryptophan. The nitrogen contents of proteose-peptone, bovine serum and heated bovine serum are shown in Table 3 and compared with nitrogen determinations reported by others. The nitrogen content of proteose-peptone preparation was somewhat variable, depending upon the state of purity. As shown, the nitrogen content of these preparations increased with time of dialysis. In general, the nitrogen contents of these proteose-peptone preparations were slightly lower than previously reported--i.e., proteose-peptone of Brunner and Thompson (13.70-13.90, 1961), component 3 of Ng and Brunner (13.1, 1967), component 5 and 8 of Kolar and Brunner (13.8, 13.9 respectively, 1968) and milk phospho-glycoprotein of Bezkorovainy (13.6, 1965).

The proteose-peptone preparation which was desalted over the P-2 column (preparation I) had the highest nitrogen content, comparing favorably with previously reported values. Heated bovine serum preparations were unusually low in nitrogen content. This was also true for a preparation which had been dialyzed for 96 hr, thus reflecting the presence of a non-dialyzable component possessing a degree of conjugation with non-proteinaceous impurities. This particular sample was highly insoluble when added to the usual acidic or basic buffer solutions.

Protein-bound hexose content was determined colorimetrically by Dubois' method (1956). The colorimetric determination of hexose involves the reaction of the carbohydrate moiety with sulfuric acid to give a furfural derivative, yielding colored condensation products when reacted with a phenolic base compound. By this method, simple sugars, oligosaccharides, polysaccharides and their derivatives including the methyl ethers with free or potentially free reducing groups are detectable.

Data in Table 4 show the hexose content of proteose-peptone and heated bovine serum. The hexose content of the former fraction was significantly higher than that reported by previous workers. One of the possible reasons might be the presence of contaminating lactose in the preparation desalted over the P-2 column. The previous workers, i.e., Thompson and Brunner (1960), Ganguli et al. (1967) and Bezkorovainy (1965), determined the hexose content by Winzler's method which used orcinol as the phenolic base. Ng (1967) compared these two methods (Dubois' and Winzler's) and found Dubois' method more sensitive. The hexose content of heated bovine serum was found to be in the range of that previously reported for proteose-peptone. But heated bovine serum seemed to contain sugars other than hexose, such as pentoses or their methyl glycosides. This was concluded from the observation that the transmission spectrogram of proteose-peptone differed from

TABLE 4.--Protein-bound hexose content of proteose-peptone and heat-stable bovine serum, and comparison of proteose-peptone hexose content between this study and other workers

Component	Proteose-peptone			Phospho-glycoprotein
	This study	Thompson and Brunner[a]	Ganguli[b]	Bezkorovainy[c]
Hexose (%)	3.36-3.53[d]	--	--	--
Hexose (%)	4.55-5.75	2.90-4.30	2.30	3.10
Method used	Dubois	Winzler	Winzler	Winzler

[a]Thompson and Brunner (1960)

[b]Ganguli et al. (1967)

[c]Bezkorovainy (1965)

[d]Heat-stable bovine serum

that of the heated bovine serum. Proteose-peptone showed a minimum transmittancy near 490 nm, whereas heated bovine serum showed an additional minimum transmittancy at 405-410 nm. Dubois et al. (1956) presented characteristic absorbancy spectra for each of the sugar classes. The hexoses and their methylated derivatives had an absorption maximum at 485 to 490 nm, whereas certain of the methylated pentoses and their methyl glycosides showed specific absorptions at about 415 to 420 nm.

The color response to the Molish test also showed difference between the proteose-peptone and heated bovine serum preparations. In this test carbohydrate is degraded to furfural and 5-hydroxymethylfurfural by sulfuric acid which combines with two moles of sulfonated naphthol to form a triarylmethane chromogen. This chromogen is further oxidized in sulfuric acid to a colored quinoid compound. Proteose-peptone formed a typical red-violet ring at the acid-solution interface, whereas heated bovine serum developed a dark-brownish purple ring. This seemed to be a color mixture of red-violet and some other color probably resulting from other carbohydrates.

Characterization of Samples

Acrylamide Gel Electrophoresis

Horizontal acrylamide gel electrophoresis was performed with both continuous and discontinuous buffer

systems. The electropherograms for the proteose-peptone, heat-stable bovine serum and normal bovine serum are shown in Figure 8A (discontinuous system) and 8B (continuous system). Usually, a higher degree of resolution was achieved by the discontinuous system than by the continuous system. However, it should be noted that the fast-moving zone--component 8--seemed to concentrate at the ion front in the discontinuous gels. And, with the continuous system, component 8 was separated into two multi-zoned areas, the faster zone (8-fast) showed two minor bands whereas the slower zone (8-slow) showed one major and two minor bands. The slow-moving zone--component 3--appeared as a single zone in both systems. Component 5 migrated as a single zone in the continuous system and resolved into two principal bands and three minor bands in the discontinuous system.

Larson and Rolleri (1955) observed three distinct boundaries attributable to the proteose-peptone fraction by free-boundary electrophoresis. Also, they noted that components 3 and 8 were further diffused and that component 5 split into several peaks with prolonged electrophoresis. By acrylamide gel electrophoresis, Kolar and Brunner (1970) observed that component 5 migrated as a single zone in a continuous buffer system, whereas a double zone was reported in starch-urea-gels (discontinuous system). Thus, polyacrylamide gel electrophoresis with a discontinuous

buffer system, as reported by Melachouris (1969), seemed to show the highest resolving power among the methods that have been reported thus far for component 5.

Gel electropherograms of heated bovine serum are represented by slot 3 in Figure 8, A and B. A satisfactory resolution of its components was not achieved by either the discontinuous or continuous system. This was a reflection of polydispersity and insolubility of the preparation. However, the electropherograms obtained by the discontinuous system were considerably clearer than those obtained by the continuous system. None of the principal serum proteins--albumin and immunoglobulins--were apparent in the heated bovine serum preparation. As seen in slot 4, Figure 8, A and B, whole bovine serum was characterized by a predominant albumin zone, accounting for 41.0% of the total blood serum protein. Bands appearing in the heated bovine serum preparation were not visible in the whole serum. Probably, the heat-stable proteins, representing minor components, were obscured by the high content of the principal proteins.

Electropherograms of heated bovine serum and proteose-peptone preparations indicated the presence of some common components.

Staining of Glycoproteins of Proteose-peptone and Heat-stable Bovine Serum Following Electrophoresis

Carbohydrate-containing proteins were detected in acrylamide gels by a modified periodic acid-Schiff technique reported by Zacharius et al. (1969). Several other methods, including Keyser's (1964), were tried. A partial success was achieved by the method of Keyser; components 5 and 8 showed negative or only weakly stained zones. In this procedure of staining, distinctive yellow zones of glycoproteins on a pink background were encountered when the gel was first immersed into the fuchsin solution. However, the weakly developed zones (components 5 and 8) disappeared during the ethanol-persulfite washing procedure, leaving only a single, heavily stained zone (component 3). This particular component has been reported to contain 17.4% carbohydrate (Ng and Brunner, 1967). The modified Keyser method reported by Zacharius et al. (1969) was relatively more satisfactory. However, it was obligatory to wash the gels thoroughly before starting the glycoprotein staining procedure in order to remove the electrophoretic buffer salts. These salts inhibit the staining of glycoprotein (Purkayatha and Rose, 1965). Also, it was notable that a more satisfactory staining of the glycoproteins was obtained with thinner gel slabs.

The glycoprotein-developed electropherograms are shown in Figure 10 (discontinuous system) and Figure 11

(continuous system). These gels were also counterstained with amido black as shown in B of Figure 10 and 11.

The three principal components of the proteose-peptone preparation--3, 5 and 8--were identified as glycoproteins (see slot 1 and 2 in Figures 10 and 11). The carbohydrate content of components 5, 8-slow and 8-fast were determined by Kolar and Brunner (1968); 1.45%, 10.27% and 2.03%, respectively. A carbohydrate content of 10.27% for component 8-slow was similar to the value (9.5%) reported by Bezkorovainy (1965) for milk whey phosphoglycoprotein.

As indicated by the relative concentration of carbohydrates in the three components, the slow-moving component 3 was most heavily colored, whereas component 5 was only lightly colored. The principal zones attributed to component 5, which were stained strongly with amido black, were stained only weakly with periodic acid-Schiff reagent. On the other hand, several zones which were well stained with PAS were not visualized distinctively with amido black. The staining patterns by amido black and PAS were also different for component 8. PAS staining showed one additional band in the slower migrating zone (8-slow) which was not detected with amido black. PAS-stained electropherograms of the proteose-peptone preparations produced by the continuous buffer system showed component 5 and 8 so indistinctly that photographic

recording was impossible. As shown by slot 4 and 5 in Figure 11, the principal PAS-stained area of component 5 was a faster migrating zone than the zone heavily stained by amido black.

The glycoprotein stained pattern of the heated bovine serum preparation is shown in slot 3, Figure 10 and 11. Only one zone was identified as a glycoprotein. This zone, when counterstained with amido black, corresponded to component 5 in the proteose-peptone preparation.

Immunological Assay

Preparation of anti-sera.--Table 2 outlines the procedure used for the production of rabbit anti-sera against proteose-peptone and bovine serum. These anti-sera exhibited strong interactions against their homologous antigens. However, the anti-bovine serum showed relatively weak interactions with proteose-peptone antigens.

Although no information exists concerning the distribution of heat-stable components in bovine serum, it was apparent that whole serum contained about five times the number of components as found in the heat-stable fraction, as observed by the interaction of anti-bovine serum with its homologous antigens and the heat stable fraction. The electropherograms (slots 3 and 4 in Figures 8A and B) of bovine serum and heated bovine serum indicated

that the heat-stable components which appeared with heated bovine serum were obscured by the principal zones (albumin and immunoglobulins) in the whole serum pattern. Therefore, possibly the antibodies to these heat-stable components were not of sufficient titer to react with a 1% proteose-peptone preparation.

Further studies will be required with anti-serum against heated bovine serum to ascertain if the heat-stable serum components are similar to the proteose-peptone.

The main difficulty and limitation of immunological assay resides with the preparation of the immune serum despite the fact that this technique represents one of the excellent tools for protein identification. Anti-serum is a biological product and as such is not a constant entity. It can never be absolutely certain that immune serum contains antibodies against all proteins of the injected antigenic mixture. Another principal difficulty encountered with the immunochemical method is the heterogeneity of antibodies. Some previous workers (Isliker, 1957) observed a striking difference in the physical properties of antibodies formed at different stages of immunization. Therefore, these variables must be considered when interpreting the results of these experiments.

Immuno-doublediffusion.--No immunological study of proteose-peptone per se has been reported. Martinez-Resa, et al. (1969), performing immunoelectrophoretic analyses with whey, observed precipitin lines which were attributed to the proteose-peptone fraction. In all, they observed about 15 antigenic proteins in whey.

Plate A in Figure 12 represents an Ouchterlony doublediffusion pattern of proteose-peptone developed with its homologous anti-serum. One intense precipitin line and two medium strength precipitin arcs were formed near and toward the antibody wells in both experiments. The position and curvature of the lines depend upon diffusion rates of the two reactants and thereby upon their molecular weight and configuration, as well as upon their relative proportions. The molecular weight range of proteose-peptone has been given at 4,100 to 200,000 (Ng and Brunner, 1967; Kolar and Brunner, 1968). On the other hand, molecular weight of rabbit antibodies range from 150,000 to 180,000 (Grabar, 1959). Therefore, proteose-peptone antigens should diffuse faster than antibodies in 2% agar gels. The concentration of the antigen added to the wells was 2% unless otherwise mentioned.

The doublediffusion patterns of proteose-peptone, bovine serum, heat-stable bovine serum and whey developed with anti-proteose-peptone are shown in Plate B of

Figure 12. One of most intense precipitin lines of proteose-peptone was continuous with lines emanating from the other three antigens. Simply stated, there appeared to be a common component between proteose-peptone and bovine serum.

The relationship between milk protein and bovine serum protein was investigated by many previous workers. Hanson (1959), for example, reported fourteen of twenty-one antigenic factors in blood serum were related to milk proteins. More specific information was obtained by Martinez-Resa et al. (1969), who characterized the whey proteins in relation to origin by the immunoelectrophoretic technique, indicating that six of fifteen whey proteins were of serum origin and the remaining nine proteins were of mammary origin. They also noted that one of the six serum related whey proteins was a glycoprotein. Judging from their observation and the results of this study, this whey glycoprotein could possibly be one of the serum components.

Plate C, Figure 12, represents a doublediffusion pattern of three different proteose-peptone preparations and a protein fraction isolated from the fat-globule membrane. Anti proteose-peptone serum was used to develop the precipitin lines. Definitely, proteose-peptone preparation 3 was missing one major component. This preparation was column-desalted, crude proteose-peptone and

showed an absence of component 3 by gel electrophoresis (slot 1 in Figure 7B). Of interest was the observation that one dense precipitin line was found near the antigen well of the membrane protein which was continuous with one precipitin line in the proteose-peptone well. This reaction developed relatively late following the development of all other precipitin lines. The water-soluble membrane protein of cow's milk was reported by Herald and Brunner (1957), who observed that it possessed heat stability and compositional characteristics quite similar to proteose-peptone. Thus, they suggested a possible relationship between the two protein systems. This present observation confirms their suggestion by providing tentative qualitative evidence. Plates A, B and C in Figure 12 were performed with template pattern A in Figure 5.

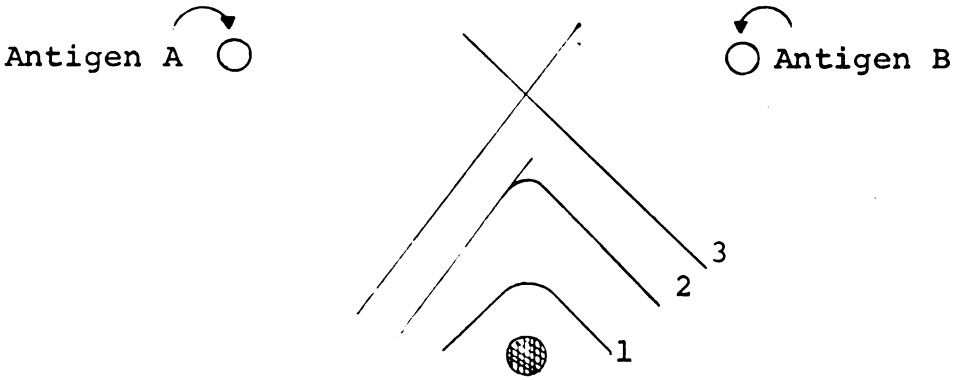
The doublediffusion pattern shown in plate D, Figure 12 (template pattern G) shows the pattern obtained when components 3, 8-slow and the heat-stable serum preparation were added to wells positioned at different distances from the antibody slot (anti-proteose-peptone). Therefore, the points of equivalence for each of the reactants were estimated roughly by using the same concentration of antigen but at different distances from the antibody. Antigen well (1) seemed to be optimum for components 3 and 8-slow, whereas antigen well (2) showed the most development for the heat-stable serum antigens. A

total of five precipitin arcs, consisting of three intense and two weakly diffused lines, were observed for component 3. This observation corresponds to that reported by Aschaffenburg and Drewry (1959), who counted six electrophoretically discernable zones in proteose-peptone prepared according to Rowland (1938a). One major component had a mobility corresponding to component 5 and the slow-moving heterogeneous component 3 was resolved into five small zones. No interaction was recorded between component 8-fast and the anti-proteose-peptone. However, four weak precipitin lines were observed in 8-slow. By gel electrophoresis in a continuous buffer system, this component was resolved into one principal and two or three minor zones (see Figure 8, plate B, slot 2). And, seven finely separated precipitin lines were observed for the heat-stable serum preparation (vs anti proteose-peptone).

Plate A of Figure 13 shows a diagrammatic representation of an immuno-doublediffusion pattern obtained when proteose-peptone, whey, heat-stable serum and bovine serum were reacted with anti-proteose-peptone-absorbed bovine serum. No precipitin lines were seen between the heat-stable serum or bovine serum antigen wells and the antibody well. However one medium dense precipitin line emanating out of the proteose-peptone well showed an identity with one of the precipitin lines in whey. One additional precipitin line was attributed to whey. This

pattern indicated that there is a component common to both bovine serum and proteose-peptone and that proteose-peptone has some additional components not of serum origin. In plate B, anti-proteose-peptone serum absorbed with homologous antigen, i.e., proteose-peptone, was developed against the same antigens as listed above. No reaction bands were anticipated for this experiment. However, as is evident in the diagram (Figure 13, Plate B), a reaction was encountered. The only explanation offered is that the absorption antigen was not added in sufficient concentrations to complex all the homologous antibodies, thus, permitting excess antibodies to react. The diffusion pattern looked similar to pattern (Figure 12, Plate B), which was formed by nonabsorbed anti-proteose-peptone, except that most of the reaction lines were absent. In this experiment, the anti-proteose-peptone serum was diluted with an equal volume of a 2% proteose-peptone solution. The pattern represented by Figure 13, plate A was developed with anti-proteose-peptone absorbed with an equal volume of 10% bovine serum. More refined experiments employing this principle should be performed.

Ouchterlony plates C and D in Figure 13 were designed for demonstrating the identity of common components in proteose-peptone and bovine serum; anti-bovine serum and anti-proteose-peptone serum were employed as antibodies. The pattern employed was as follows:



Anti-serum against either A or B

Three possible patterns, consisting of lines 1, 2 and 3, are predictable: 1--identity of antigens, 2--cross-reacting antigens, and 3--different antigens.

Doublediffusion patterns (plate C, Figure 13) show identities for three common components between proteose-peptone and bovine serum against anti-proteose-peptone serum. The three continuous precipitation lines indicate this relationship. However, not a single precipitin line was shown between proteose-peptone and anti-bovine serum. As discussed in a previous section, it was postulated that a 1% solution of proteose-peptone was not concentrated enough to form visible precipitates with the anti-bovine serum used in this study. In plate D, the same arrangement of samples was employed except that heat-stable serum was used in place of bovine serum. Again, three common components were found between the proteose-peptone and heat-stable bovine serum. In this plate one weak precipitin line was formed between

proteose-peptone and anti-bovine serum. Two cross-reacting antigens were also found between proteose-peptone and bovine serum as indicated by the formation of a spur at the junction of two lines. However, it was noticed that the cross-reacting precipitin lines between bovine serum and proteose-peptone appeared as a continuum, indicating an identity.

Doublediffusion patterns of various antigens developed with anti-bovine serum are represented diagrammatically in Figure 14. A diagram of plate A shows the precipitin lines that appeared in the immunodiffusion of proteose-peptone, whey, heated bovine serum and bovine serum against anti-bovine serum. One indistinct band was formed with the proteose-peptone which was continuous with all antigens. At least ten precipitin lines were formed with bovine serum. Hanson (1959) found a total of twenty-one antigenic factors in bovine serum when displayed against anti-bovine serum following electrophoresis in agar gel. Only two precipitin lines appeared between the heat-stable bovine serum and anti-bovine serum. Thus, it was estimated that bovine serum contained approximately five times as many antigenic substances as heat-stable serum. Therefore, antibodies against the heat-stable components in bovine serum which are potentially reactive with components of proteose-peptone may exist in concentrations too low to form reaction lines. The same

four antigens as used for developing plate A were diffused against anti-bovine serum absorbed with proteose-peptone, see plate B. The resulting reaction pattern was less complex than plate A; some components related to proteose-peptone were eliminated. One of the two precipitin bands apparent in plate A remained in the heat-stable serum. Thus, not all of the heat-stable bovine serum components are found in proteose-peptone. No reaction appeared in the proteose-peptone well and several of the lines disappeared from the bovine serum and whey samples. However, the exact number of disappearing lines could not be determined by the doublediffusion technique.

In plate C, proteose-peptone and purified component 8-slow (Kolar, 1967) were added to antigen wells at two different concentrations and distances, see pattern type D, Figure 5. Two weak lines were observed for components 8-slow and proteose-peptone at 2% concentration, whereas four medium dense lines and three strong lines were observed at 4% concentration of components 8-slow and proteose-peptone, respectively. Identities were established between two bands of component 8-slow and proteose-peptone. Plate D shows the immunodiffusion pattern of purified component 3 (Ng, 1967) against anti-bovine serum (using template pattern B). One intense band in a 4% concentration of component 3 showed an identity with one line of component 8-slow. The concentration of component

3 was increased to 8% in plate G. Still only one intense precipitin line appeared. The purity of these isolated components (3, 8-slow and 8-fast) was established by acrylamide gel electrophoresis (Figure 15 B, C, D). No purified sample of component 5 was available. Portions of the gel corresponding to the principal electrophoretic zone of component 5 were taken from unstained, companion gels and subjected to doublediffusion as shown in plate E, Figure 14. Figure 15, plate A, slots 1, 2, 3 and 5 show acrylamide gel patterns obtained with different concentration of proteose-peptone by a continuous buffer system. The gel was sampled as indicated by the excised plugs. Not a single precipitin line was developed from these reactions.

Acrylamide gel electropherograms of heated bovine serum indicated that components with relative electrophoretic mobilities corresponding to component 5 were present. The unexpected failure of component 5 to react with anti-bovine serum was attributed to either its low concentration in the excised gel section or to a low titer in the anti-bovine serum.

The microdiffusion pattern represented by plate H, Figure 14 shows the precipitin lines observed when three different concentrations of components 8-fast and 8-slow were reacted with anti-bovine serum. Here again, 8-slow shows two reaction zones, whereas no reaction

between 8-fast and anti-bovine serum was apparent. Consequently, a more detailed study with purified components 5 and 8-fast should be undertaken before concluding that they are not components of bovine serum.

The immuno-doublediffusion technique is limited in its resolving capacity for a complex mixture, thus positive identification of components in an antigenic mixture is sometimes difficult to achieve. Precipitin zones can represent more than one reaction complex or when either component of a reaction pair is present in large excess, a multi-precipitin zone can occur. Thus, the data obtained for the more complex mixtures in this study were only qualitative indications of composition.

Immunoelectrophoretic analysis.--The immunoelectrophoretic technique overcomes some of the shortcomings encountered with immuno-doublediffusion. The principal advantage results from the electrophoretic resolution of antigens prior to doublediffusion. The immunoelectrophoretic analyses reported here utilized only the anti-proteose-peptone serum as the developing antibody system. One set of experiments was attempted to illustrate immunoelectropherograms of several heterogeneous antigenic systems developed with anti-bovine serum. As illustrated in Figure 16, Plate B, no precipitin lines were formed between the proteose-peptone (2%) and the

anti-bovine serum. However, in the reverse case, when bovine serum (2%) was treated as the antigenic system against anti-proteose-peptone serum (Figure 16, plate A), four precipitin lines were observed. Thus, it was concluded that the antibody titer of the proteose-peptone components in the anti-bovine serum was too low to permit the use of this anti-serum.

When developed with anti-proteose-peptone serum, a 2% solution of the heat-stable serum preparation showed one more precipitin line than those observed for the bovine serum. As stated previously, the heat-stable components in bovine serum, which are the counterpart of the proteose-peptone fraction of whey, accounted for about one-fifth of the serum proteins. Thus, the pattern of their immunological response need not be similar. When bovine serum (10%) and proteose-peptone (2%) were compared immunoelectrophoretically (plate D, Figure 16), at least five of the nine or ten precipitin lines developed with anti-proteose-peptone serum appeared to be common components. A more definite assessment of identities between proteose-peptone and bovine serum might be obtainable by employing anti-bovine serum absorbed with proteose-peptone as the developing antibody system. However, in these experiments no precipitin lines were observed, supporting the previously stated suggestion

that the proteose-peptone titer in the anti-bovine serum was too low to be useful.

Immunoelectrophoretic analysis of proteose-peptone by means of its homologous immune serum showed a reaction pattern of eight to ten separate precipitin arcs (see Figure 16, plates B, C, D and E). Characteristics of immunoelectropherograms, i.e., number, shape, position of precipitin arcs, varied slightly depending on the technique employed. Factors such as antigen concentration, distance between antigen and antibody wells, and template patterns were variables. Also, the proteose-peptone components were not resolved distinctly by the electrophoretic migration.

The immunoelectropherograms of a 20% solution of whey proteins developed with anti-proteose-peptone serum is shown in Figure 16, plate C; a 2% solution of proteose-peptone is on the opposite side of the antibody trough. The immunoprecipitin spectrum of whey was more complex than that of the proteose-peptone showing fifteen and nine precipitin lines, respectively. Whey showed several lines in the cathodic position of the gel which were not present in the proteose-peptone pattern. The relationship between the antigenic components in proteose-peptone and heat-stable serum is shown by the immunoelectropherogram in Figure 16, plate E. At least five of the reaction zones seemed to be common. These patterns (Figure 16) were

based on template pattern K, Figure 5 except for plate E which was based on pattern H.

As shown in Figure 17, plate A, the electrophoretic patterns of 10% whey and 10% bovine serum showed similarity in number, shape and position of precipitation arcs when developed with anti-proteose-peptone serum.

The immunoelectropherograms of purified component 3 developed with anti-proteose-peptone serum is presented in Figure 17, plate B; with proteose-peptone on the other side of the antibody trough. Five dense precipitin lines, same as the number developed by immunodiffusion, are apparent near the edge of anti-serum trough. Most of the elongated precipitin lines in the proteose-peptone pattern seem to correspond to the component 3 pattern. However, the number and shape of precipitin zones seem to differ slightly, probably as a result of a variation in concentration. The concentration of component 3 in the purified preparation was higher than that of component 3 in the proteose-peptone fraction. A distribution study of component 3 in whey and casein revealed that component 3 was absent in casein, being present only in whey (Kolar and Brunner, 1970; Ng, et al., 1970). Thus, it was suggested that component 3 was of serum origin. The doublediffusion pattern of component 3 developed with anti-bovine serum showed this relationship. Immunoelectropherograms of heated bovine serum and component 3

developed with anti-proteose-peptone serum showed some similarities (See Figure 17, plate D). Seven precipitin lines are apparent for the heated bovine serum--same as the number developed by immuno-doublediffusion--and six for component 3--one more line than those shown by double-diffusion. At least four of these lines appear to be common to both antigen systems.

Immunoelectropherograms of purified component 8-slow and proteose-peptone developed with anti-proteose-peptone serum are shown in Figure 17, plate C. Component 5 could not be analyzed immunoelectrophoretically since a purified specimen was unavailable. The analyses shown in Figure 17 were performed with template pattern H except plate D which was performed with template pattern K (see Figure 5).

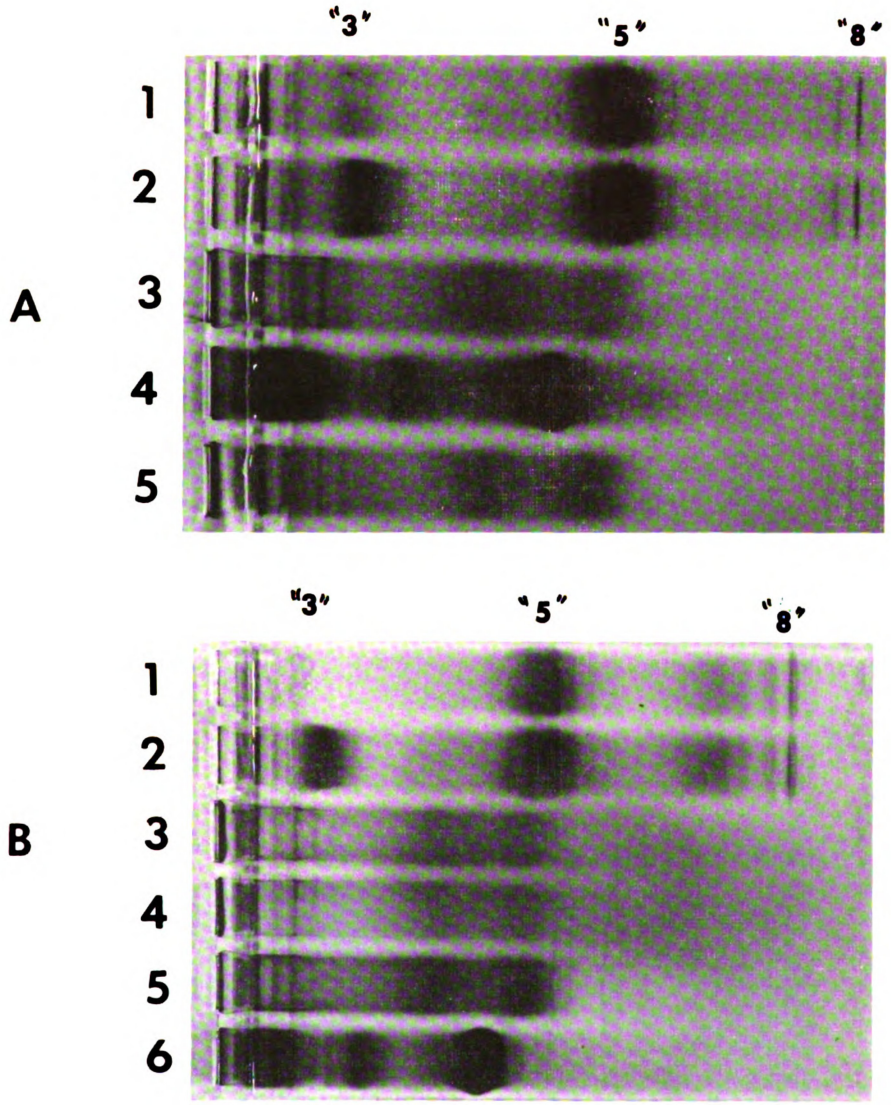


Figure 7.--Acrylamide gel electropherograms obtained with the discontinuous buffer system. Plate A: Slot 1, proteose-peptone (preparation 1 desalted over Bio-Gel P-2 column); Slot 2, proteose-peptone (dialyzed); Slot 3 and 5, heat-stable bovine serum; Slot 4, bovine serum. Plate B: Slot 1, proteose-peptone (preparation 2 desalted over Bio-Gel P-2 column); Slot 2, proteose-peptone (dialyzed); Slots 3 and 4, heat-stable bovine serum (dialyzed); Slot 5, heat-stable bovine serum (desalted over Bio-Gel P-2 column); Slot 6, bovine serum.

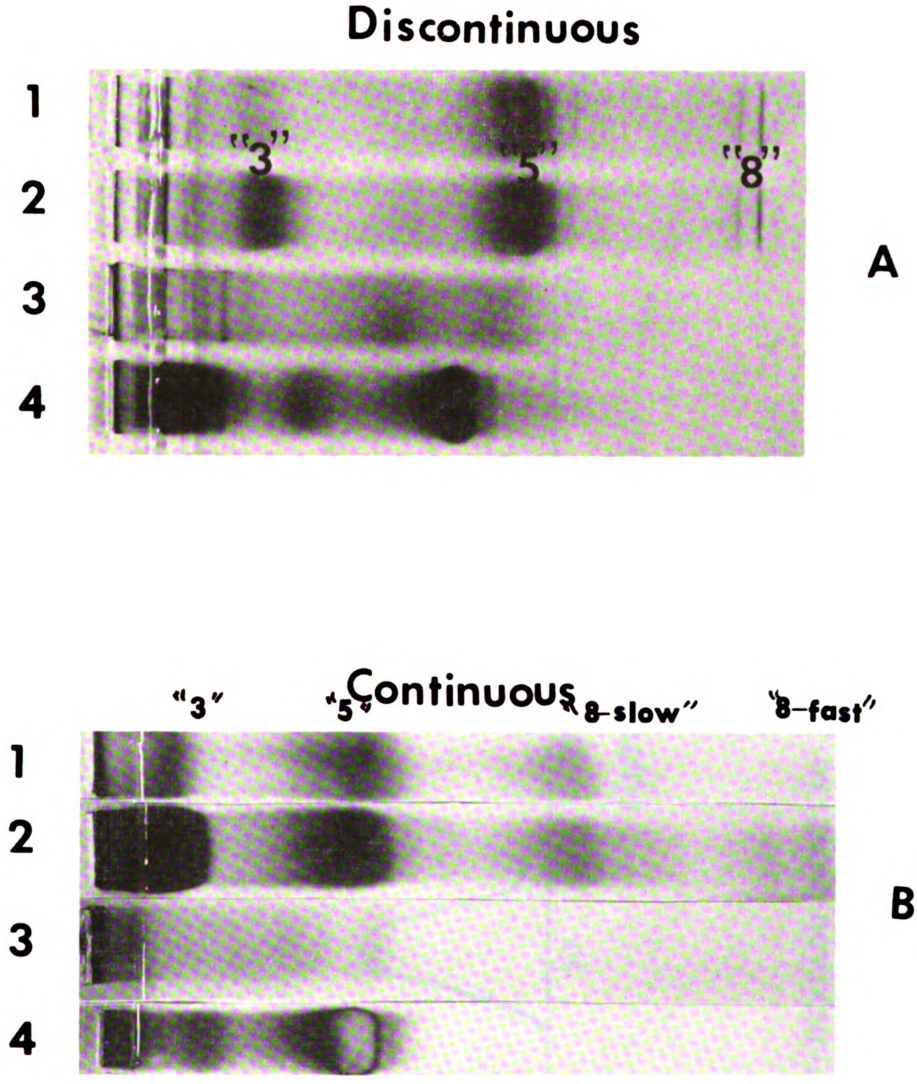


Figure 8.--Acrylamide gel electropherograms obtained with the discontinuous buffer system (A) and with the continuous buffer system (B). Plate A: Row 1, proteose-peptone (desalted over Bio-Gel P-2 column); Row 2, proteose-peptone (dialyzed); Row 3, heat-stable bovine serum; Row 4, bovine serum. Plate B: Similar to samples in Plate A.

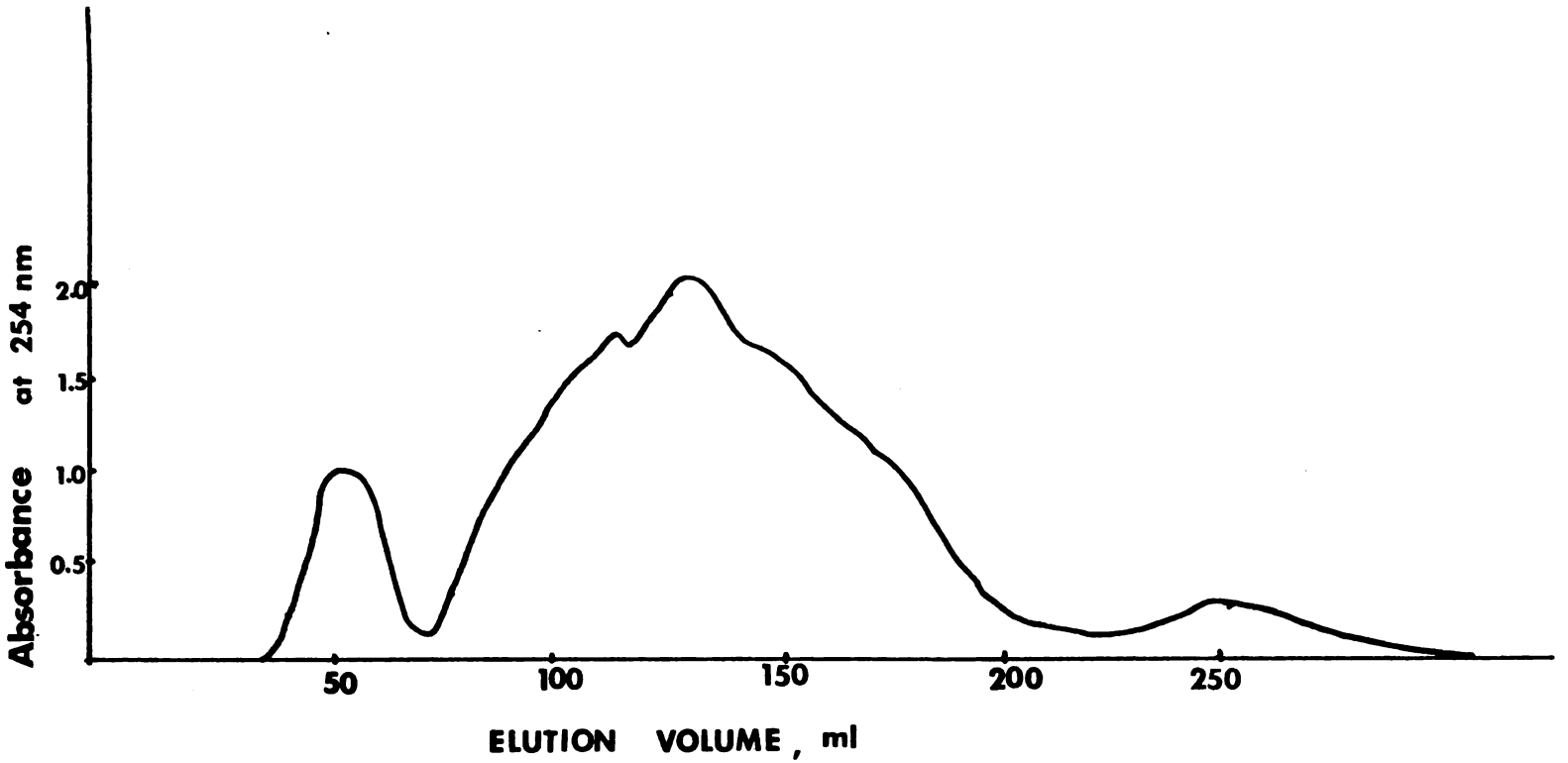


Figure 9.--Elution profile resulting from the passage of a proteose-peptone fraction through a Bio-Gel P-2 column equilibrated with ammonium acetate, pH 4.6. Eluate was monitored at 254 nm. Peak 1, proteose-peptone; Peak 2, lactose, salts and other soluble components.

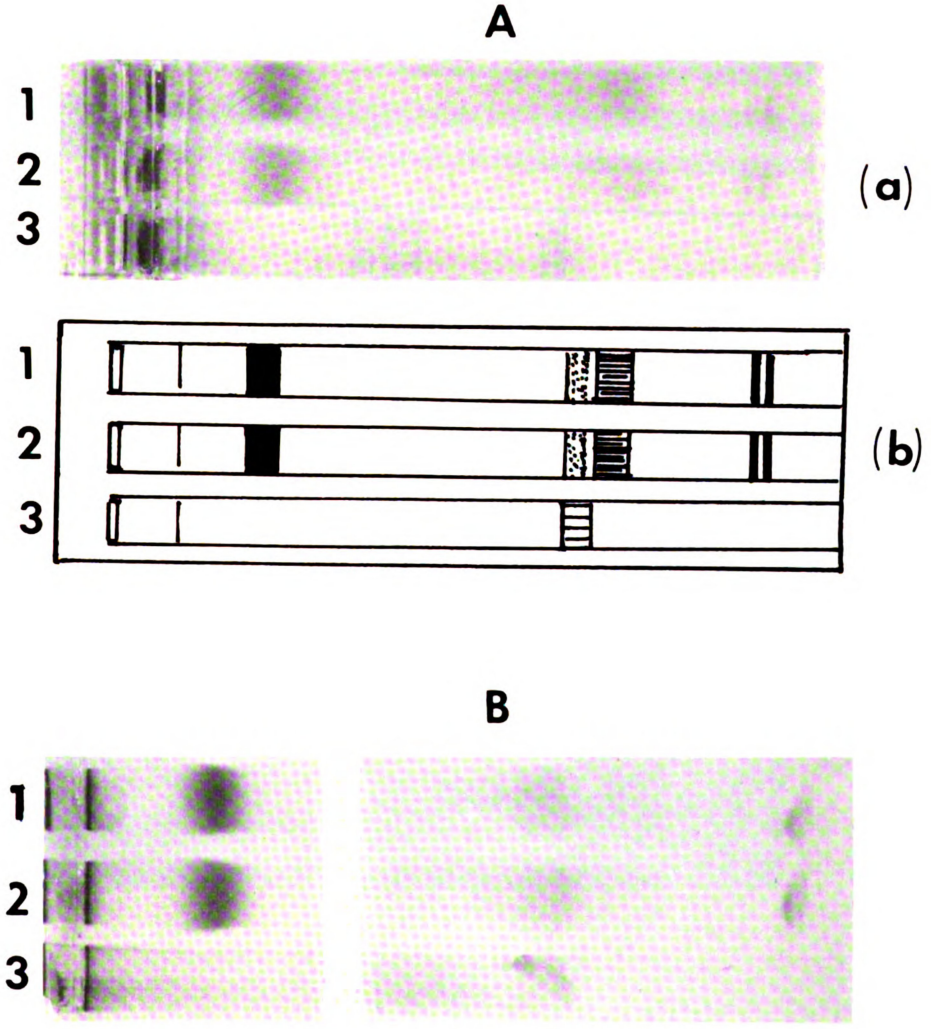


Figure 10.--Acrylamide gel electropherograms (discontinuous buffer system) stained with periodic acid-Schiff reagent (Plate A: a--photograph; b--diagram). The gel was counter-stained with amido black (Plate B). Slots 1 and 2, proteose-peptone; Slot 3, heat-stable bovine serum.

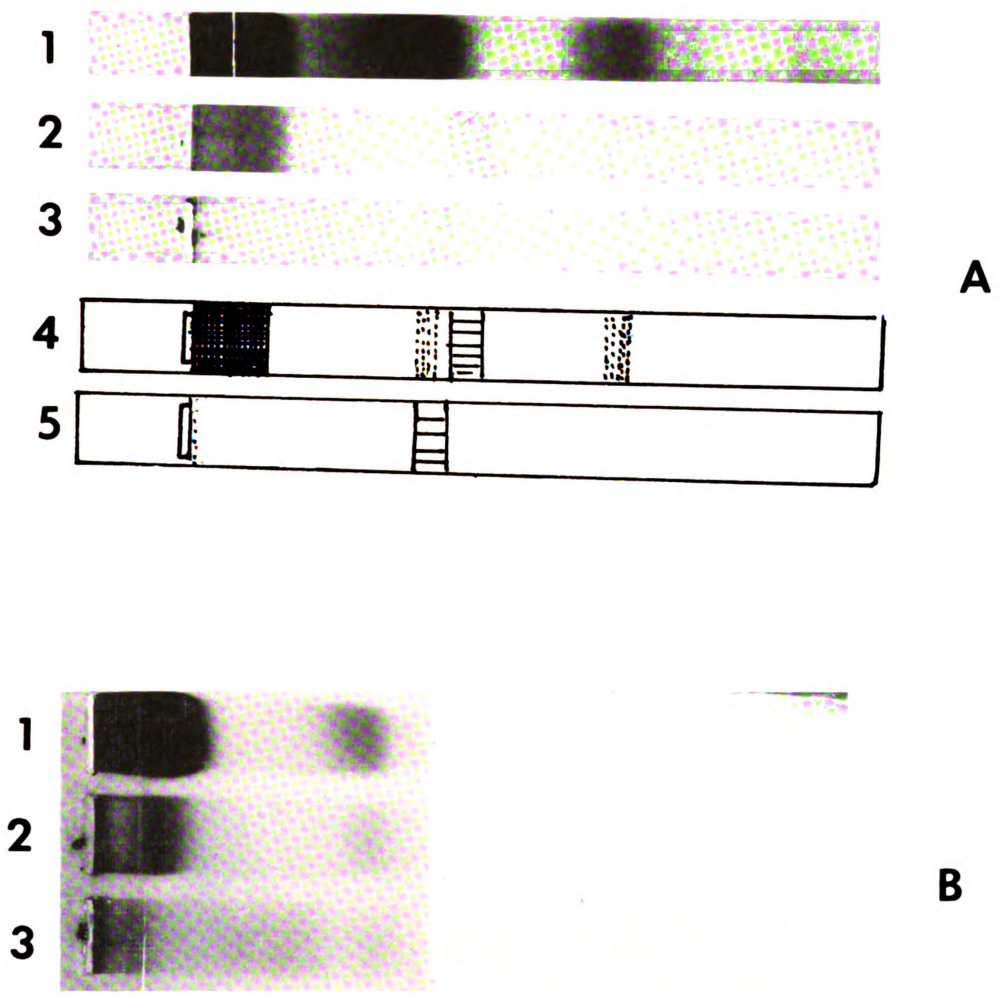


Figure 11.--Acrylamide gel electropherograms (continuous buffer system) stained with periodic acid-Schiff reagent (Plate A except row 1): Slot 1, proteose-peptone stained with amido black prior to glycoprotein staining; Slot 2, proteose-peptone; Slot 3, heat-stable bovine serum; Slot 4, diagram of slot 2; Slot 5, diagram of slot 3. Plate B: Counterstained with amido black. Slots 1 and 2, proteose-peptone; Slot 3, heat-stable bovine serum.

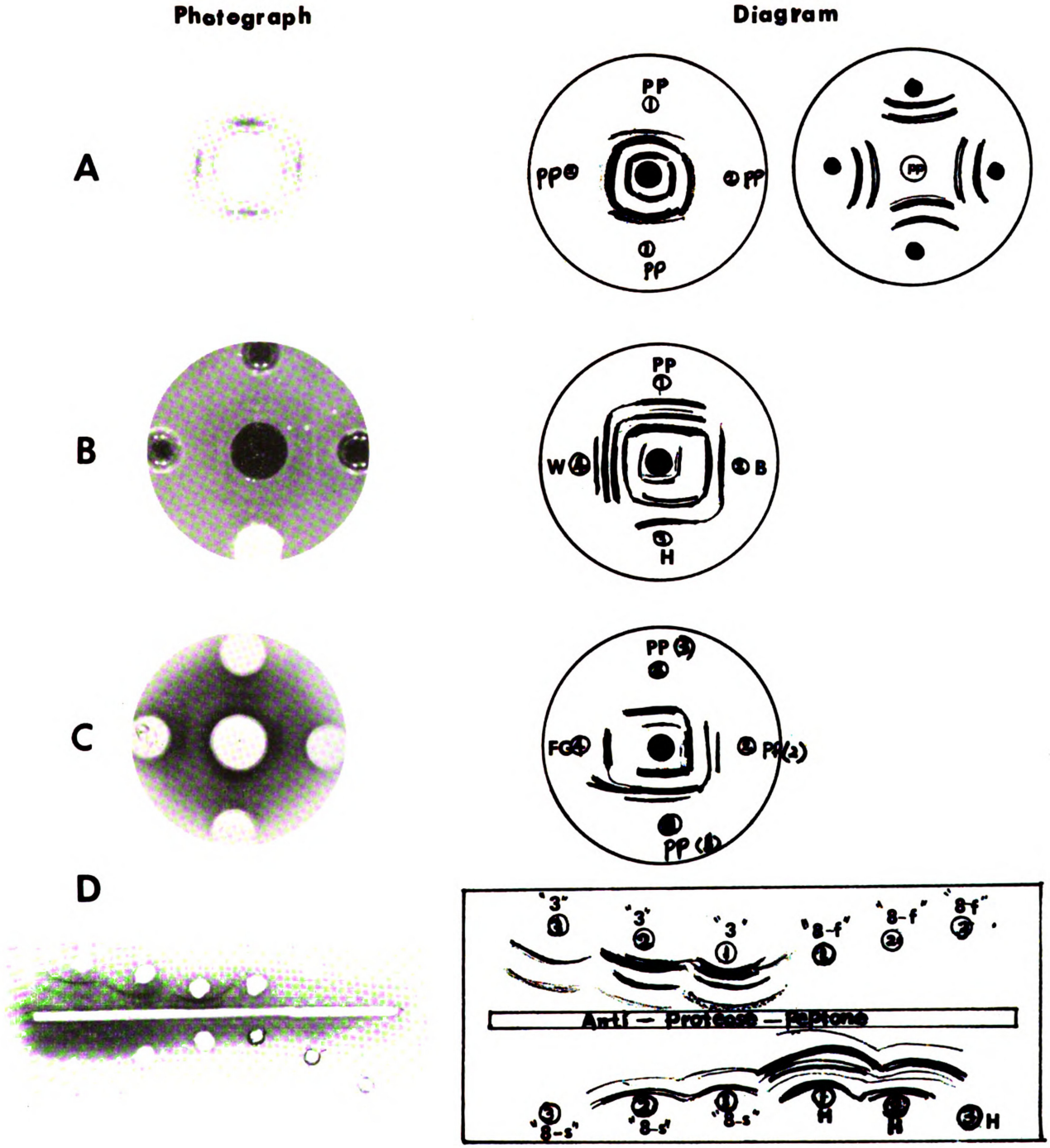


Figure 12.--Immuno-doublediffusion patterns of homologous and heterologous antigens developed with anti-proteose-peptone immune serum. Antigen designations are: whey (W), fat-globule membrane (FG), component 3 ("3"), 8-slow (8-s) and 8-fast (8-f).

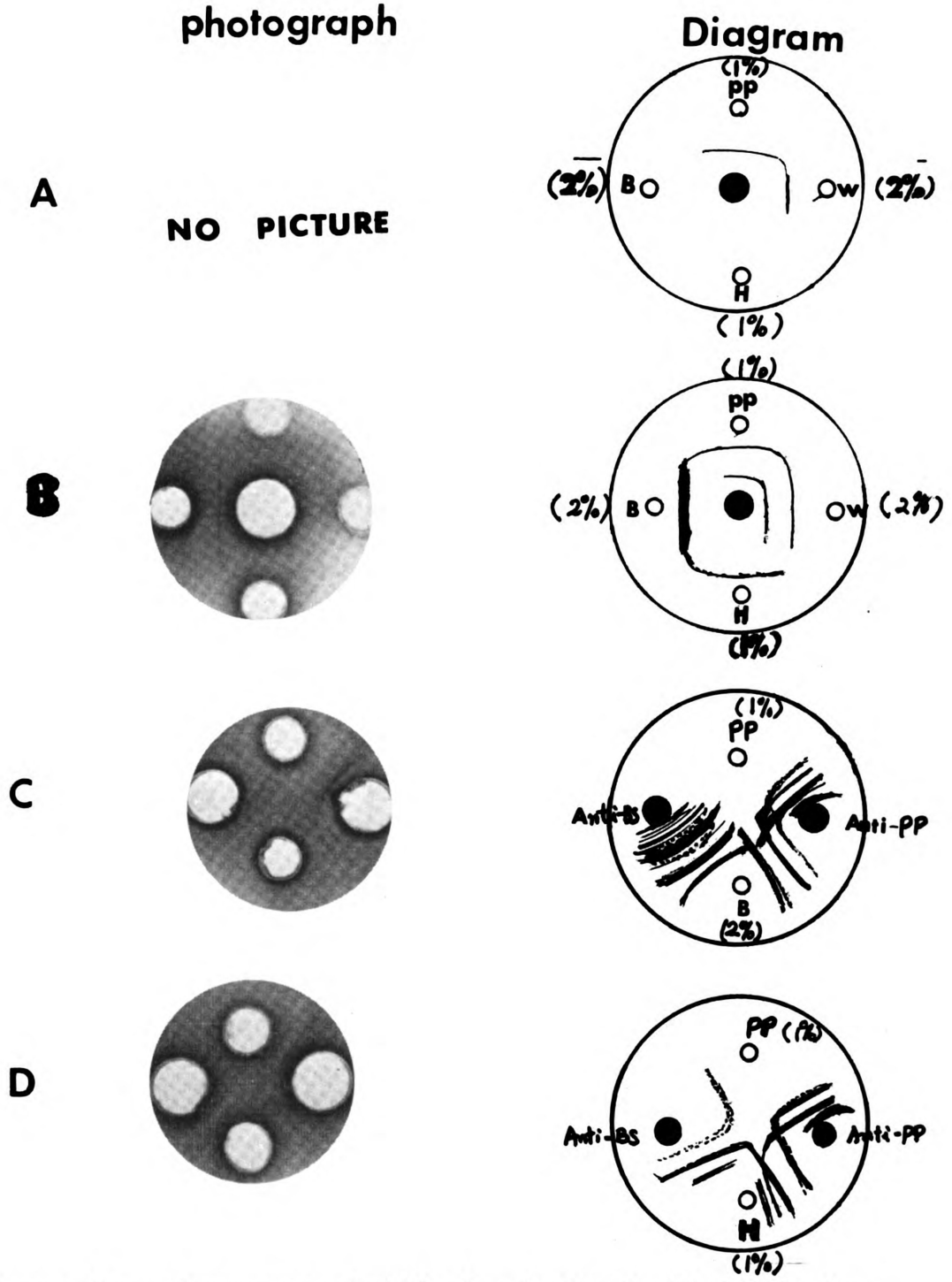


Figure 13.--Immunoprecipitin bands by doublediffusion. Antigen designations are: proteose-peptone (PP), whey (W), bovine serum (B) and heated bovine serum (H). Anti-sera designations are: A-anti-proteose-peptone immune serum absorbed with bovine serum. B-anti-proteose-peptone immune serum absorbed with proteose-peptone; C and D-anti-proteose-peptone immune serum (Anti-PP), anti-bovine serum (Anti-BS).

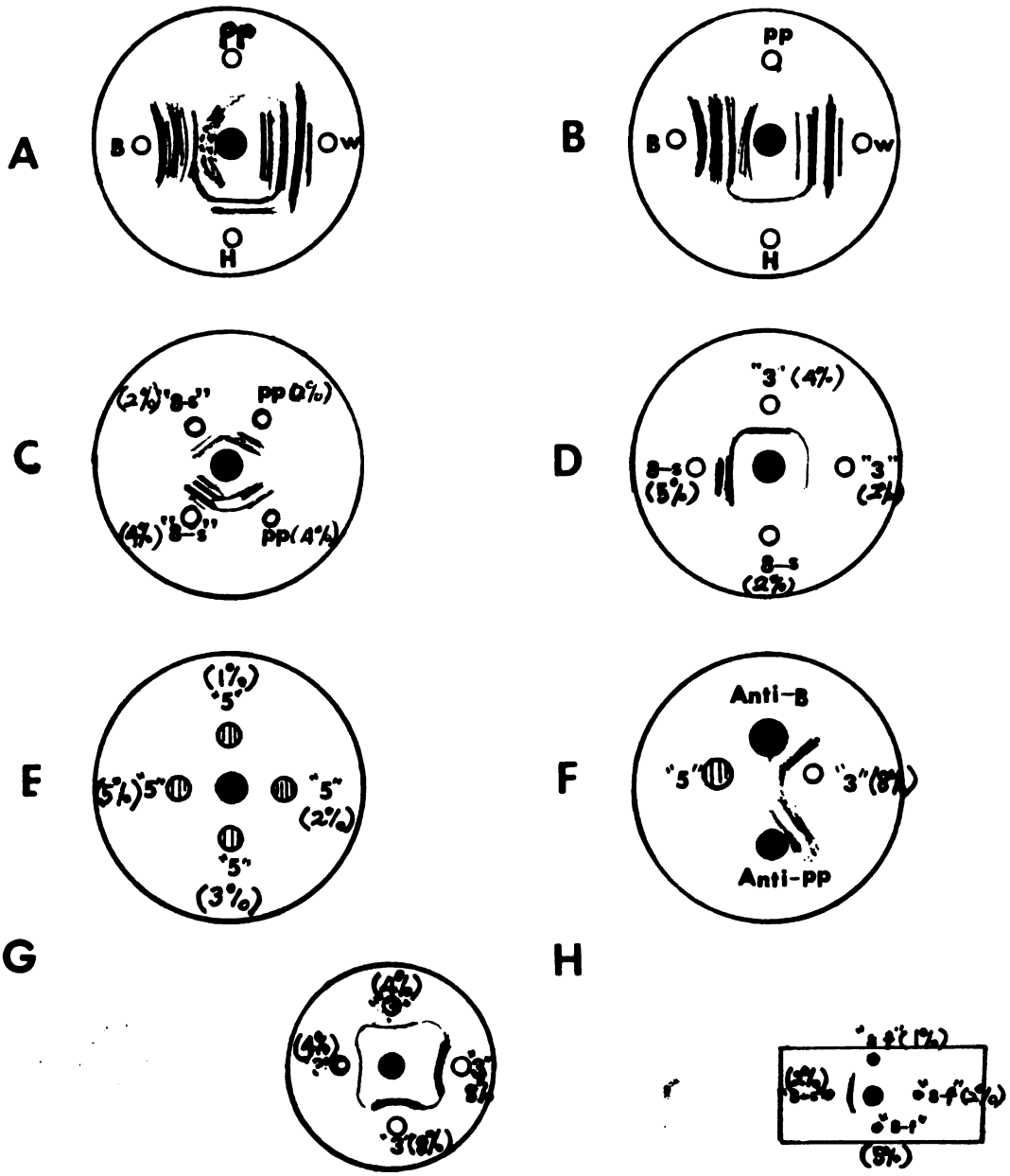


Figure 14.--Immuno-doublediffusion patterns. Antigen designations are: proteose-peptone (PP), bovine serum (B), heated bovine serum (H), whey (W), component 8-slow (8-s), component 8-fast (8-f), component 3 ("3") and sliced gel for component ("5",0). Anti-sera designations are: anti-bovine serum (A,C,D, E,G and H); anti-bovine serum absorbed proteose-peptone (B); anti-bovine serum (Anti-B) and anti-proteose-peptone (Anti-PP) as (F).

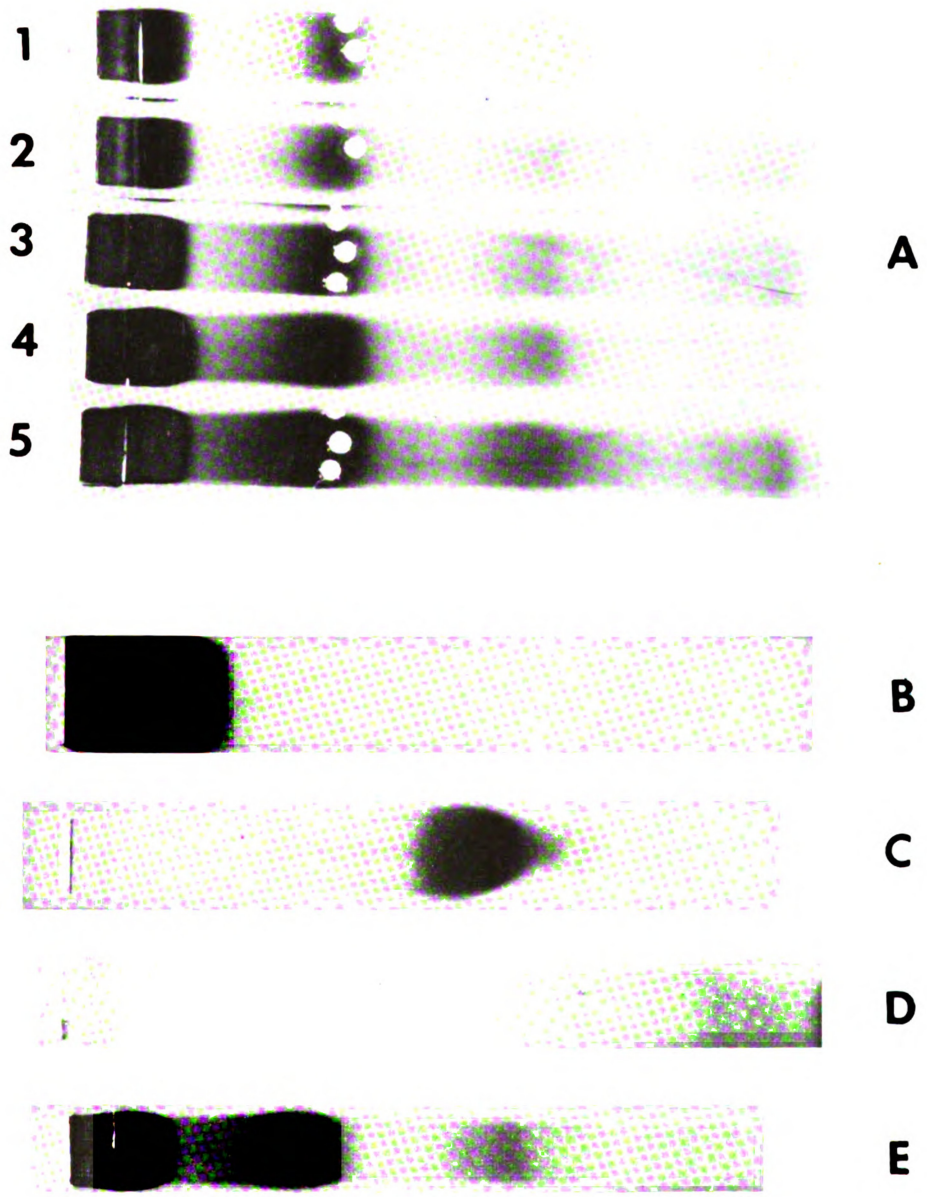


Figure 15.--Acrylamide gel electropherograms obtained with a continuous buffer system. Plate A: Slots 1 and 2, 2% proteose-peptone; Slot 3, 3% proteose-peptone; Slot 4, 4% proteose-peptone; Slot 5, 5% proteose-peptone. Circles indicate excised gel section corresponding to location of component 5. Plate B: Isolated component 3. Plate C: Isolated component 8-slow. Plate D: Isolated component 8-fast. Plate E: 4% proteose-peptone.

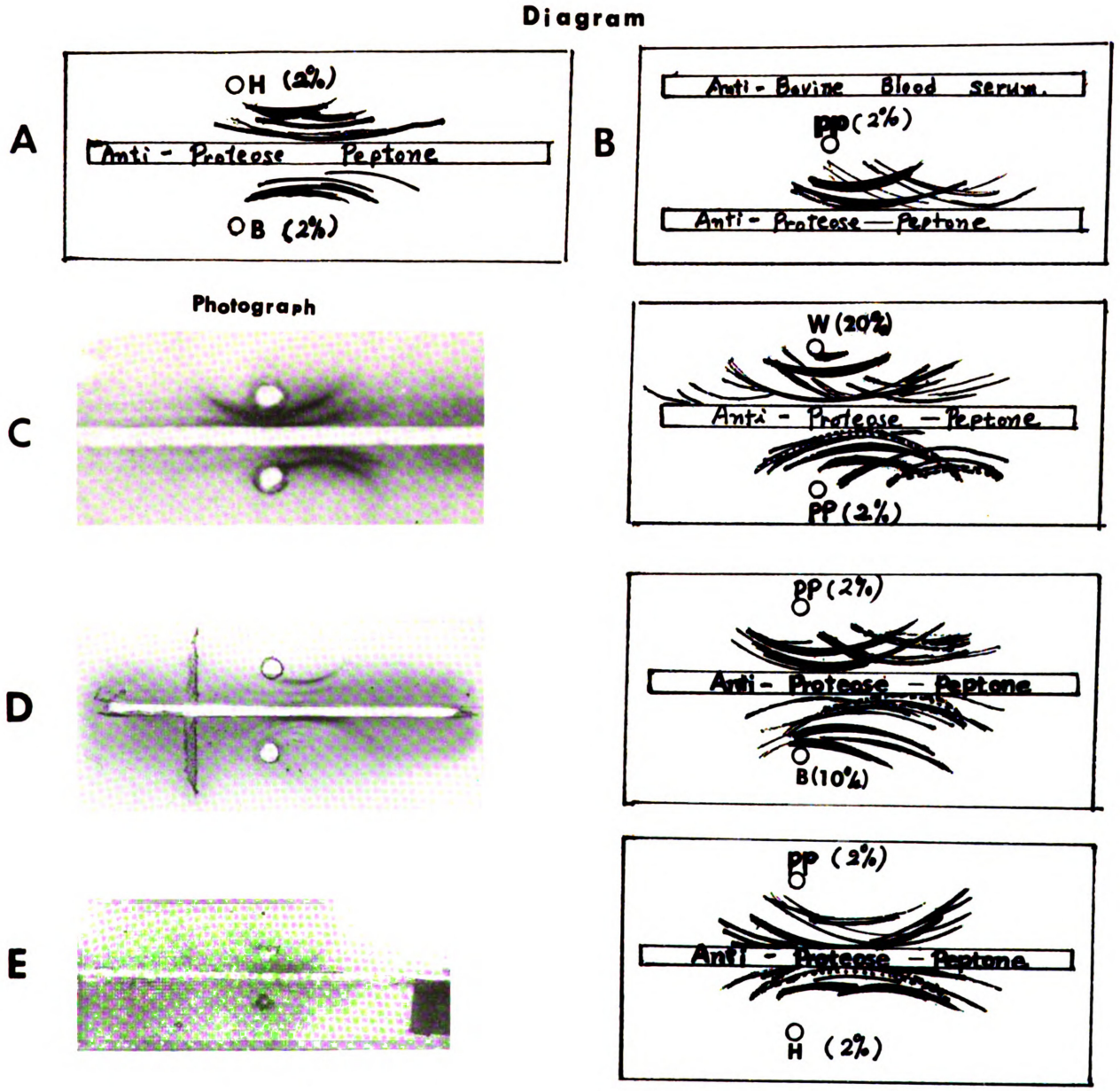


Figure 16.--Immunoelectropherograms of proteose-peptone (PP), whey (W), bovine serum (B) and heated bovine serum (H) developed with anti-proteose-peptone immune serum.

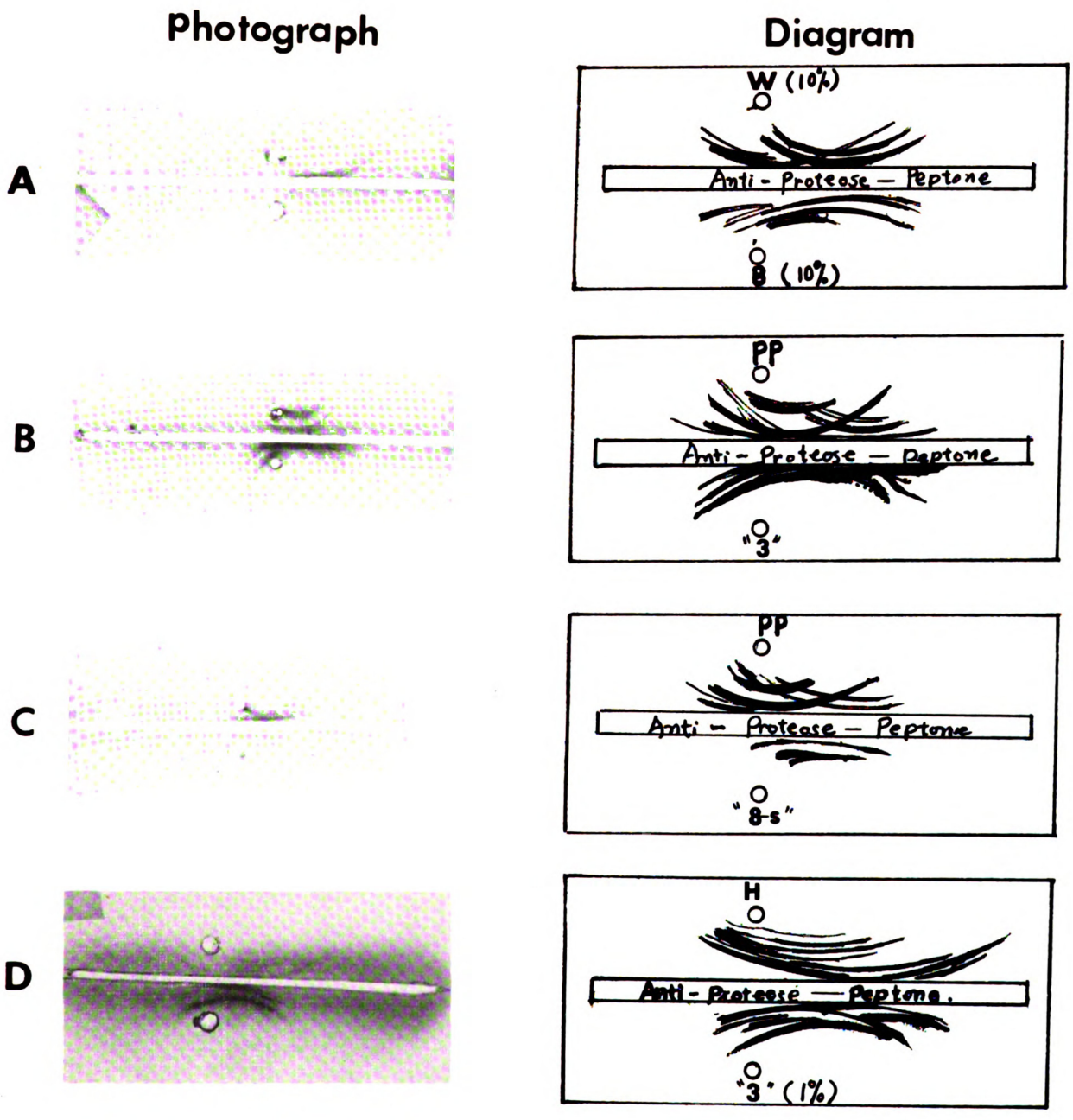


Figure 17.--Immunoelectropherograms of proteose-peptone (PP), whey (W), bovine serum (B), component 3 ("3") and component 8-slow ("8-s") developed with anti-proteose-peptone immune serum.

CONCLUSION

The experimental evidence presented herein permits the conclusion that at least two of the proteose-peptone components, possibly component 3 and 8-slow, are similar to components native to bovine serum, and that the proteose-peptone fraction contains additional components which are not of serum origin. However, more detailed studies should be conducted with high titer anti-sera against proteose-peptone, bovine serum and heat-stable bovine serum combined with the more refined immunoelectrophoretic absorption technique to differentiate between similar and dissimilar components in these three fractions.

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APPENDIX

COMPOSITION OF JENNESS AND
KOOPS' BUFFER

Solution I

The following quantities of salts were made to 900 ml with deionized water:

KH_2PO_4	1.58 g
K_3citrate$\cdot H_2O$	0.51 g
Na_3 citrate$\cdot 5H_2O$	2.12 g
K_2SO_4	0.18 g
Mg_3 citrate$\cdot H_2O$	0.50 g
K_2CO_3	0.30 g
KCl	1.08 g

Solution II

A 1.32 g of $CaCl_2 \cdot 2H_2O$ was dissolved in 50 ml of deionized water.

Solution II was added very slowly to Solution I while the mixture was stirring virogously, and the content made up to one liter with deionized water.