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ELECTROPHORETIC CHARACTERIZATION OF THE PROTEOSE-PEPTONE FRACTION OF BOVINE MILK

By

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ABSTRACT

ELECTROPHORETIC CHARACTERIZATION OF THE PROTEOSE-PEPTONE FRACTION OF BOVINE MILK

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Classical acid-soluble and heat-stable proteose-peptone specimens were prepared from skimmilk, casein-free, centrifuged serum, and micellar casein. As many as 38 protein-stained zones were evidenced in PAGE-discontinuous. Sixty-two zones could be accounted for by excising zones from 7.5% PAG and reelectrophoresing them in 17.5% PAG.

Twenty-two zones, ranging in molecular weight from <3000 to ~90,000, were revealed by SDS-PAGE, with a large number of species $\leq$31,000 daltons.

As many as 23 zones, ranging in pI from 3.8 to 5.3, were demonstrated in IEF-PAG.

Zones stained for phosphorus and carbohydrate verified that phosphoproteins, glycoproteins, and phosphoglycoproteins exist in this fraction.

Previous observations that component 3 is of serum origin and that components 5 and 8 are casein-associated and distributed between the casein micelle and the serum were substantiated. All three principal components were confirmed to be electrophoretically heterogeneous.

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INTRODUCTION

The proteose-peptone fraction of cow's milk is an uncharacterized group of acid-soluble (pH 4.6) and heat-stable (95 C for 30 min) proteins found in the plasma. This fraction is believed to consist primarily of minor caseins that are distributed between the casein micelle and the serum. Certain components are believed to be of serum origin. Electrophoresis has been the primary means of characterization of this fraction, with only four components--3, 5, 8-slow, and 8-fast--being identified with suspected heterogeneity in each. The presence of phosphorus and carbohydrate has been detected.

Zonal electrophoresis in polyacrylamide gel simultaneously exploits differences in molecular size and molecular net charge for purposes of fractionation. The mobility of a macromolecule in polyacrylamide gel electrophoresis (PAGE) is directly proportional to its intrinsic charge, the pH of the buffer system employed, the magnitude of the electric field applied, and inversely proportional to the frictional resistance encountered by the macromolecule due to its size and the density of the gel.

The possibility exists that a larger protein, more highly charged, and a relatively smaller protein, less highly charged, may migrate at the same rate, and appear as a single zone. Thus the

appearance of a single zone in PAGE should not be interpreted as unequivocal evidence of homogeneity. Different gel concentrations should be used to determine whether a single zone is actually homogeneous (Hedrick and Smith, 1968). Increasing the percentage of monomer in PAGE, while keeping the ratio of monomer to crosslinker constant, reduces the pore size of the gel matrix and enables one to separate zones that may be moving together even though they differ in size and charge.

Discontinuous buffer systems in PAGE have been demonstrated to afford maximal resolution due to the ultra-thin starting zones achieved (Ornstein, 1964).

Zonal electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE), in which the proteins have been heated to aid in SDS binding, has been demonstrated to separate proteins solely on the basis of size (Shapiro, Vinuela, and Maizel, 1967; Weber and Osborn, 1969). This is accomplished by the proteins unfolding, binding the same amount of the highly negatively charged SDS (1.4 g SDS/g protein), assuming a rod-like form which is directly proportional to their molecular weight (Reynolds and Tanford, 1970), and moving through the gel matrix as simulated cylinders (Svasti and Panijpan, 1977).

Electrophoresis in polyacrylamide gels containing carrier ampholytes, in which a linear pH gradient is produced, has been demonstrated to separate proteins solely on the basis of charge. The proteins migrate to, and reside, at their isoelectric points (Finlayson and Chrambach, 1971). This procedure is referred to as isoelectric focusing in polyacrylamide gel (IEF-PAG).

Cognizant of the ability of each of the above electrophoretic methods to separate proteins on entirely different characteristics,

the author utilized PAGE in a discontinuous buffer system, SDS-PAGE, and IEF-PAG to better assess the number of components comprising the proteose-peptone fraction. By isolating the proteose-peptone components from skimmilk, casein-free centrifuged serum, and micellar casein the distribution of these components in the milk protein system was investigated. The presence of phosphorus and carbohydrate in this fraction was examined by differential staining of the electrophoretic zones for these substances.

REVIEW OF LITERATURE

Osborne and Wakeman (1918) were among the first workers to suggest the presence of an acid-soluble and heat-stable protein in milk. They observed that some proteinaceous material still remained in solution after the albumins and globulins of acid whey were heat-denatured. They were uncertain as to whether this protein was indigenous to milk or an artifact resulting from the heat treatment.

Palmer and Scott (1919), using tannic acid to precipitate protein from casein-free, heat-denatured whey, postulated that lactalbumin and lactoglobulin were not the only proteins present in a casein-free filtrate of milk.

Kieferle and Gloetzl (1930), and Kieferle (1933) employed phosphotungstic acid to precipitate soluble proteins from heat-coagulated milk, classifying these components as proteoses and peptones.

Jones and Little (1933) reported the presence of considerable amounts of proteose in milk, designated as the material precipitated by 10% trichloroacetic acid (TCA), but not by 5% TCA.

Moir (1931) reported that approximately 70% of the soluble proteins of casein-free filtrate of milk were removed by heat coagulation.

Rowland (1938a) designated the proteose-peptone as a protein fraction which did not precipitate at pH 4.7 after heating skimmilk at 95 C for 30 min but was precipitated by 12% TCA. He (1938b) reported the nitrogen distribution of normal milk as follows: 78.5% casein N, 9.2% albumin N, 3.3% globulin N, 4.0% proteose-peptone N, and 5.0% non-protein N. 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 minute amounts of proteose resulted from the hydrolysis of protein.

Harland and Ashworth (1945) noted that casein obtained by saturating skimmilk with NaCl accounted for more nitrogen than conventionally obtained casein. The whey from this fractionation, when acidified to pH 3.0, contained 17.7% less nitrogen than that released by Rowland's precipitation method. In addition, 95% of the whey proteins in this preparation were coagulated by heating to 95 C for 10 min compared with 76% by Rowland's method.

Aschaffenburg (1946) isolated a surface-active material, which he called sigma proteose, by heating skimmilk to 90-95 C for 15 min, followed by coprecipitation of the casein and denatured serum proteins by acid or rennet coagulation. Treatment of this fraction with one-half saturated ammonium sulfate resulted in a precipitate, which was called sigma proteose. This fraction contained significantly less nitrogen than other principal milk proteins. In free-boundary electrophoresis in phosphate buffer at pH 8.0, three components were seen in decreasing order of mobility, accounting for 10.5%, 82.5%, and 7.0%, respectively, of the total protein in sigma proteose, demonstrating this fraction's heterogeneity.

Ogston (1946) examined this fraction by ultracentrifugation, finding that it was heterogeneous with 49% of the material consisting of a single, well-defined constituent of molecular weight 4900 and a component comprising 11% of the fraction with a molecular weight of 23,000. The remaining 40% of the fraction was unaccounted for.

Weinstein, Duncan, and Trout (1951) isolated a protein fraction from heated, rennet whey by a procedure similar to that of Aschaffenburg, calling it the "minor-protein fraction," capable of being photosensitized to produce the solar-activated flavor of homogenized milk. From elementary analysis, the minor-protein fraction was different from sigma proteose. The nitrogen content of this fraction, 10%, was low compared to the 13.95% for Aschaffenburg's sigma proteose. In the Tiselius cell, at least two components were present in the minor-protein fraction, with the isoelectric zone of the major components at pH 3.7 to 4.4, based on electrophoretic mobilities at various pH values (Weinstein, Lillevik, Duncan, and Trout, 1951).

Gordon, Jenness, and Geddes (1954) reported that both casein and whey depressed the loaf volume of bread when used in the dough formulation. Jenness (1959) reported that component 5 isolated from the proteose-peptone fraction of raw milk was the heat-labile loaf-volume depressant. Volpe and Zabik (1975) isolated a loaf-volume depressant, which they attributed to proteose-peptone component 5, from acid whey and from whey ultrafiltrate. Sodium dodecyl sulfate polyacrylamide gel electrophoresis yielded a molecular weight of 14,000-15,000 for this component. Fuchsin-sulfite dye staining of the gel component indicated that the loaf-volume depressant was a glycoprotein.

Larson and Rolleri (1955), studying the effect of heat treatment on free-boundary electrophoretic mobilities of whey proteins, attributed peaks 1, 2, 4, 6, and 7 to euglobulin, pseudoglobulin, α-lactalbumin, β-lactoglobulin, and serum albumin, respectively, with peaks 3, 5, and 8 being proteose-peptones. Increasing heat treatments were employed, until at 95 C for 30 min, there were only peaks 3, 5, and 8 remaining, which accounted for 4.6%, 8.6%, and 5.7%, respectively, of the whey proteins. The electrophoretic mobilities of components 3, 5, and 8, calculated from the descending boundaries, were -2.9, -4.5, and -7.9×10^{-5} cm^2 $volt^{-1}$ sec^{-1}, respectively. Since proteose-peptone components appeared in the electrophoretic pattern of unheated whey, Larson and Rolleri suggested that the proteose-peptone fraction was indigenous to milk.

Jenness (1957) isolated a proteose-peptone fraction, precipitated by saturation with NaCl, 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. Over 90% of this material had an electrophoretic mobility of -4.5×10^{-5} cm^2 $volt^{-1}$ sec^{-1} at pH 8.6 (Veronal) and contained 1.2-1.3% phosphorus. The fraction was soluble in the presence of $CaCl_2$ and not affected by rennin. This is interesting in that in Jenness' procedure, the proteose-peptone components were obtained from fractions associated with micellar casein, while previously, the proteose-peptone components were shown to be present in the whey protein fraction.

Aschaffenburg and Drewry (1959) noted six bands in paper electropherograms of acid filtrates derived from heat-treated skimmilk.

The prominent band corresponded to peak 5 and the five minor bands corresponded to peak 3. The same six bands were found in unheated skimmilk, further indicating their presence in native milk. By salting-out with sodium sulfate at a concentration of 12 g/100 ml, the heterogeneous fraction could be separated from the other whey proteins present in the casein-free filtrate at pH 4.6. 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, possibly indicating the presence of carbohydrates.

Thompson and Brunner (1959) were the first workers to demonstrate that the proteose-peptone fraction contained glycoproteins. They identified hexose, hexosamine, fucose, and sialic acid in several minor proteins of milk--the soluble membrane protein, Weinstein's minor-protein fraction, and proteose-peptone. A high hexose and sialic acid content was a common characteristic of these fractions. They suggested that the glycoproteins of the proteose-peptone fraction might originate from blood serum.

Brunner and Thompson (1961) reported chemical composition and physical parameters of five minor protein fractions, namely: Rowland's proteose-peptone, Aschaffenburg's sigma proteose, Jenness' component 5, Weinstein's minor-protein fraction, and the soluble fat globule membrane protein. The chemical composition of these five protein fractions were very similar in that they contained low amounts of sulfur-containing amino acids, contained carbohydrate, and were high in phosphorus. Some similarity was observed in their electrophoretic and ultracentrifugal properties. They concluded that a common component existed in all the fractions.

Marier, Tessier, and Rose (1963) indicated that 17% to 28% more sialic acid was present in proteins precipitated with 12% TCA 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 TCA.

Bezkorovainy (1965) isolated orosomucoid and M-2 glycoproteins from bovine serum and colostrum whey, and M-2 glycoprotein from whey. Milk whey contained no orosomucoid and only trace amounts of the M-2 glycoprotein. Furthermore, both milk and colostrum whey contained a phosphoglycoprotein which had practically no absorption at 280 nm, thus reflecting the low concentration of aromatic amino acid residues. Physical and chemical properties of this phosphoglycoprotein corresponded to the major component of the milk proteose-peptone fraction. Bezkorovainy concluded that close relationships could exist between colostrum glycoproteins and the proteose-peptone fraction.

Ganguli, Gupta, Joshi, and Bhalerao (1967) determined the sialic acid and hexose contents of the proteose-peptone fraction of milk. Proteose had higher concentrations of sialic acid and hexose than the corresponding proteose-peptone sample. Even though the casein fraction contributed the largest share of sialic acid to the sialic acid distribution in milk proteins, the concentration of sialic acid in the proteose-peptone fraction was four times that found in the casein fraction. The sialic acid found in the proteose-peptone and proteose fractions was identified as a neuraminic acid derivative, similar to that found in k-casein.

Joshi, Ganguli, and Bhalerao (1971) found that proteose-peptone of colostrum differed significantly in its concentration, its sialic

acid content, its molecular size, and its electrophoretic pattern from the proteose-peptone in milk. They contended that proteose-peptone in milk is likely to originate from mammary function whereas proteose-peptone in colostrum probably appears from the blood. Joshi, Ganguli, and Bhalenao (1971) observed that blood serum proteose-peptone has higher concentrations of sialic acid compared to that of milk proteose-peptone. The proteose-peptone from the blood shows more marked similarity with colostrum proteose-peptone than proteose-peptone from milk. They concluded that proteose-peptone in milk does not come from the blood.

Ganguli's group (1966, 1967, 1968a,b) made an extensive study of the effect of storage, trypsin action, rennet action and heat treatment on the proteose-peptone content of milk. The proteose-peptone content increased upon heating milk to 25 C, presumably derived from other milk protein fractions such as casein and the whey proteins. Upon heating at 30 C and 37 C, some proteose-peptone apparently had also undergone degradation since the non-protein nitrogen increased while the proteose-peptone decreased. 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 milk nearly doubled its proteose-peptone content. They suggested that a proteose-peptone-like material and non-protein fractions were released from casein, increasing 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. By gradually removing casein from milk by ultracentrifugation, the corresponding milk serum showed a linear increase in its proteose-peptone content. Utilizing a synthetic model system comprising proteose-peptone and micellar casein, the proteose-peptone level decreased upon heating whereas a similar system containing α-lactalbumin and β-lactoglobulin in place of micellar casein did not affect the proteose-peptone level. They postulated that a possible heat-induced interaction existed between micellar casein and proteose-peptone.

Joshi and Ganguli (1972a) treated κ-casein with 2-mercaptoethanol, using 8% TCA to precipitate the proteose-peptones, and found an increase in the proteose-peptones. They concluded that proteose-peptones could arise as a result of a selective cleavage of κ-casein at the disulfide bridges by indigenous reducing agents in the milk or the mammary glands.

Joshi and Ganguli (1972b) employed gel filtration chromatography to separate the proteose-peptone into fractions of different molecular weights, the leading peak evidencing the presence of sialic acid.

Bezkorovainy, Nichols, and Sly (1976) isolated proteose-peptone from both human and bovine milk. The proteose-peptone from bovine milk evidenced molecular weights of 30,000, 18,000, and 12,000 in SDS-PAGE.

Brunner's group has done the most detailed work on the distribution and composition of the proteose-peptone fraction. Kolar and Brunner (1965) found that component 8 was present in both casein micelles and in whey, and was a tenacious contaminant of κ-casein

preparations and a leading zone in discontinuous starch-urea gel electropherograms. Kolar and Brunner (1968) further isolated components 5 and 8 from both heated and unheated skimmilk. Component 8 was fractionated into components 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. Components 8-fast and 8-slow contained relatively higher concentrations of phosphorus. All three components were void of cysteine and cystine, and contained low concentrations of methionine.

Kolar and Brunner (1969) reported that components 5 and 8 existed in equilibria between micellar casein and the serum, whereas component 3 was not present in micellar casein. Ng, Brunner, and Rhee (1970) substantiated this convention by isolating lacteal serum component 3 from both heated and unheated skimmilk. Component 3 was characterized by its high content of carbohydrates and low content of sulfur-containing amino acids.

Physical and chemical parameters of the proteose-peptone fraction derived from Kolar (1967) and Ng (1967) are listed in Table 1.

Kang (1971) observed that the proteose-peptone fraction accounted for 18-25% of the whey proteins. Component 3 migrated as a single zone in both continuous and discontinuous polyacrylamide gels. Component 5, homogeneous in continuous gels, was resolved into five closely migrating zones when examined with a discontinuous buffer system. Component 8, migrating as two very close zones near the ion front in discontinuous gels, was resolved into multizonal areas--8-slow and 8-fast--in continuous gels. Double diffusion immunoelectrophoresis

Table 1. Physical and chemical parameters of components 3, 5, 8-slow, and 8-fast of the proteose-peptone fraction.[a]

Constituent	Component 3[b]	Component 5[b]	Component 8-slow[b]	Component 8-fast[b]
Nitrogen (%)	13.1	13.8	12.3	13.3
Phosphorus (%)	0.5	1.0	2.0	2.4
Hexose (%)	7.2	0.9	4.5	1.4
Hexosamine (%)	6.0	0.2	2.5	0.3
Sialic acid (%)	3.0	0.3	3.3	0.4
Electrophoretic mobility (cm^2 $volt^{-1}$ sec^{-1} x 10^{-5})	-3.8	-4.8	-9.2	-9.3
Isoelectric pH	3.7	-	-	3.3
$S^\circ_{20,w}$	4.0	1.2	1.4	0.8
M°_w	200,000	14,300	9900	4100
$D^\circ_{20,w}$	1.8	-	-	-

[a]Data from Kolar (1967) and Ng (1967).

[b]Prepared from heated skimmilk.

demonstrated homology between some of the proteose-peptone components and those of bovine serum.

EXPERIMENTAL

Chemical Analysis

Nitrogen

Nitrogen analyses were performed according to the semi-micro Kjeldahl method in which the ammonia is steam-distilled into 4% boric acid (Swaisgood, 1963). Selenium dioxide and cupric sulfate were the catalysts employed. Tryptophan was used as a standard, with an average recovery of 94.1%.

Physical Methods

Preparation of Proteose-Peptone Specimens

Five gallons of raw, uncooled milk was obtained from Holstein cows of the Michigan State University dairy herd. The milk was brought up to 37 C and separated immediately. Approximately four liters of the skimmilk was heated in a 95 C water bath for 40 min. After this time the milk was promptly cooled to 20 C. One normal HCl was added until pH 4.6 was reached. The milk was left to coagulate overnight at 4 C. The coagulum and serum was filtered through Whatman #1 filter paper. The filtrate was collected, dialyzed, pervaporated, lyophilized, and then stored at 0 C. Figure 1 depicts the isolation procedure used to prepare proteose-peptone from skimmilk.

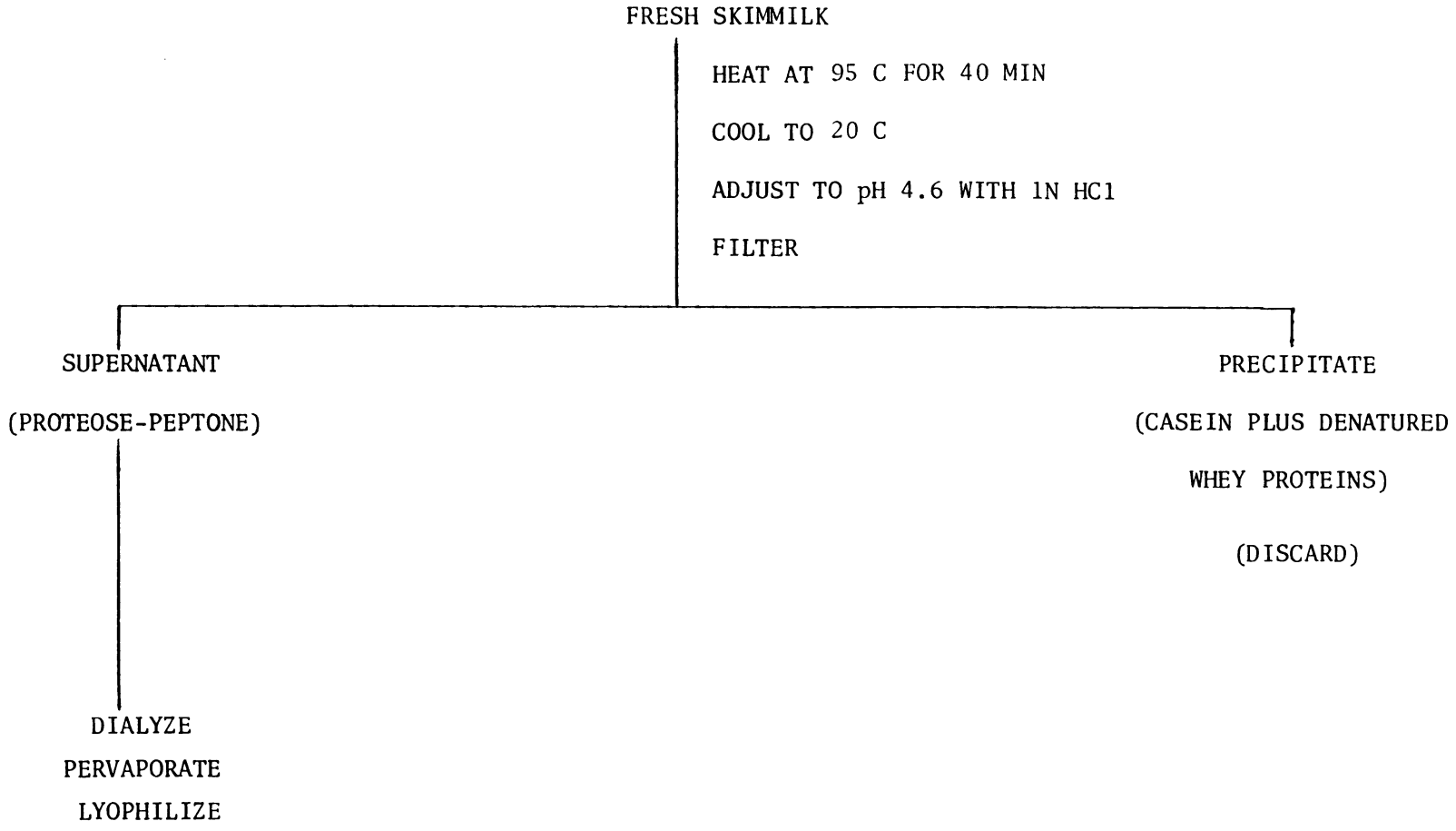


Figure 1. Procedure for the preparation of proteose-peptone from skimmilk.

The remaining four gallons of skimmilk was cooled to 20 C and centrifuged at 105,000 x g for 50 min. The supernatant was decanted, heated at 95 C for 40 min, cooled to 20 C, and acidified to pH 4.6 with 1N HCl. Centrifugation at 1000 x g for 20 min was performed for clarification purposes. The supernatant was decanted, dialyzed, pervaporated, lyophilized, and then stored at 0 C. Figure 2 portrays the isolation procedure used to prepare proteose-peptone from milk serum.

The pellet from the 105,000 x g centrifugation was collected, redispersed two times in Koops' simulated milk ultrafiltrate, and then heated at 95 C for 40 min. After cooling to 20 C, the caseins were isoelectrically precipitated by adjustment to pH 4.6 with 1N HCl. The solution was left to further coagulate overnight at 4 C. The coagulum and serum was filtered through Whatman #1 filter paper. The filtrate was collected, dialyzed, pervaporated, lyophilized, and then stored at 0 C. Figure 2 shows the isolation procedure for preparation of proteose-peptone from micellar casein.

Polyacrylamide Gel Electrophoresis in Glass Tubes

All electrophoretic studies were performed in 6 mm I.D., 2 mm walled, and 75 mm length glass tubes. The tubes were detergent washed, immersed in chromic acid, treated with Photoflo, and rinsed in distilled, deionized water after use. The acrylamide and bisacrylamide used were of the highest purity grade or were recrystallized from acetone. The acrylamide to bisacrylamide ratio was kept in a 19:1 ratio to yield 5% crosslinked gels, which afford minimal pore size at that total gel concentration (Rodbard, Levitou, and Chrambach, 1971). All polymerized gels were not used until at least 18 hours had transpired after

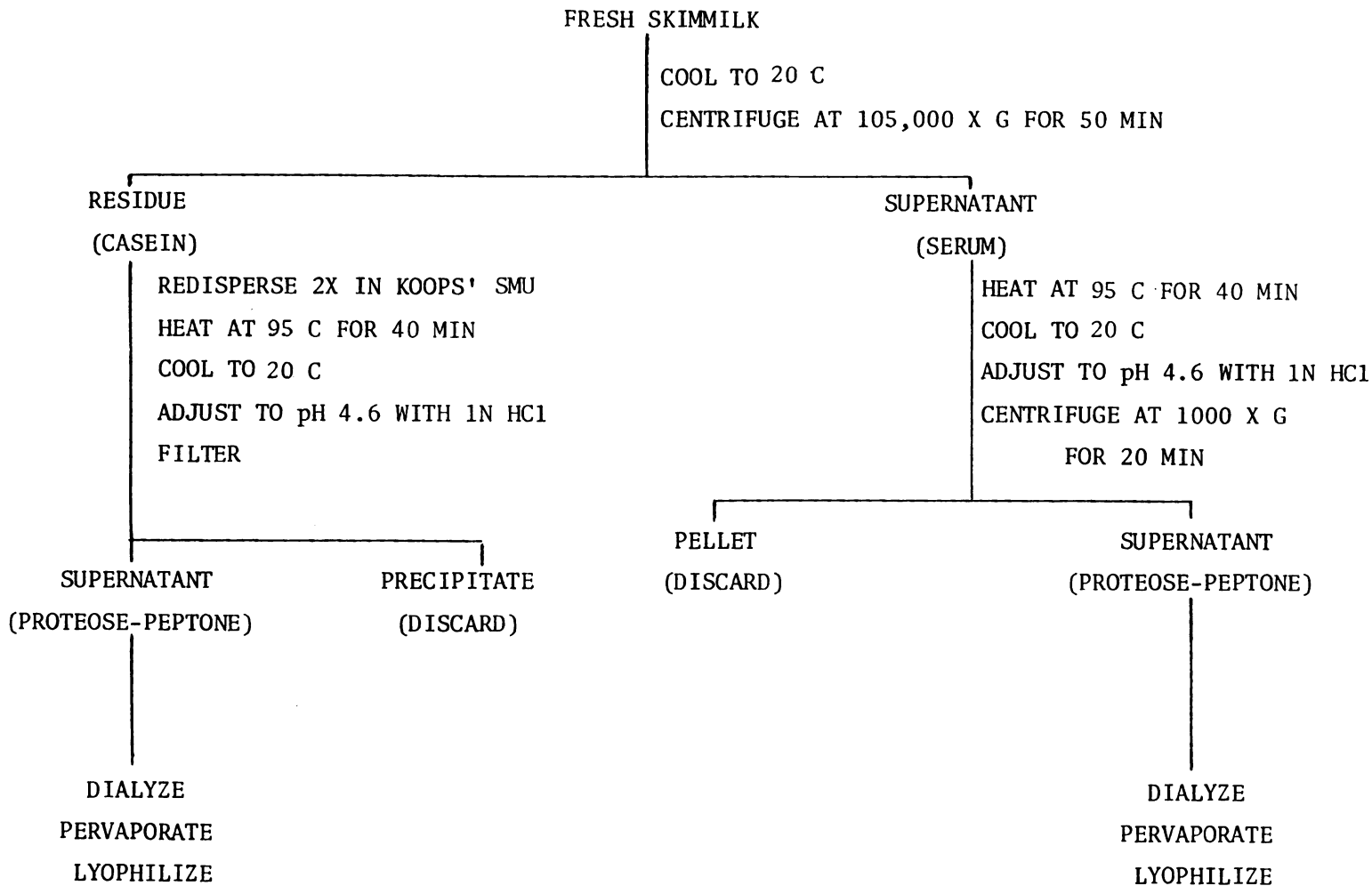


Figure 2. Procedure for the preparation of proteose-peptone from micellar casein and serum.

polymerization to insure that all gels had achieved the same polymerization state. Electrophoresis was performed in a jacketed Buchler electrophoresis apparatus that was cooled with tap water.

Polyacrylamide Gel Electrophoresis in a Discontinuous Buffer System

Polyacrylamide gel electrophoresis in a discontinuous buffer system was performed according to the method of Melachouris (1969). Modifications employed were the following:

1. A 1/10 dilution of running buffer with distilled, deionized water.
2. Protein staining was by Coomassie Brilliant Blue R (Weber and Osborn, 1969) or Coomassie Brilliant Blue G (Reisner, Nemes, and Buchalty, 1975).
3. Five percent, 7.5%, 10.0%, 12.5%, 15.0%, and 17.5% gels were employed.

Polyacrylamide Gel Electrophoresis in Gradient Pore Gels

Gradient gels, utilizing the discontinuous buffer system of Melachouris (1969), of 10-25% and 15-30% were used as a means of characterization. A sucrose gradient of 2-15% was used to stablize the polyacrylamide gradient.

The less concentrated gel solution in 2% sucrose was added to the left-hand chamber of the gradient maker, while the more concentrated gel solution in 15% sucrose, was added to the right-hand chamber. After equilibration, mixing was begun, the outlets opened, and the gradient dispensed into the gel tubes. The linear gradient was pushed up into

the apparatus containing the gel tubes with a 60% sucrose solution containing bromophenol blue.

The rest of the electrophoretic procedure was as that for single pore-size gels.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate electrophoresis in polyacrylamide gel was performed according to the method of Weber and Osborn (1969). Modifications employed were the following:

1. Protein samples were dissolved in 3% SDS, 1% 2-mercaptoethanol, in .01M phosphate buffer, pH 7.2. Incubation was at 100 C for 5 min.
2. Five percent, 7.5%, 10.0%, 12.5%, and 17.5% gels were employed.

The molecular weight standard proteins used were the following: BDH standard molecular weight proteins (14,300, 28,600, 42,900, 57,200, 71,500 and 53,000, 106,000, 159,000, 212,000, and 265,000) which are synthetically crosslinked, RNA polymerase (*E. coli*) β, β'-subunits (155,000 and 165,000), bovine serum albumin (68,000), L-glutamic dehydrogenase (53,000), ovalbumin (43,000), chymotrypsinogen (25,700), β-lactoglobulin B (18,276), ribonuclease (13,700), and insulin (3396 and 2337).

Isoelectric Focusing in Polyacrylamide Gel

Isoelectric focusing in polyacrylamide gel was performed according to the method of Josephson (1972). The following modifications were employed:

1. Gels of 12.5% total concentration and 1% crosslinkage were used.
2. Sucrose was eliminated from the gel formulation.
3. Protein was layered at the gel surface, rather than incorporated into the gel.
4. Staining was by the method of Reisner et al. (1975).

Ampholine of pH 3.0-5.0 and pH 4.0-6.0 was used. The pH gradient in the gel was determined by slicing four identical gels into 5 mm sections, determining the pH of these sections by penetrating the gel surface with a microelectrode, and plotting pH versus distance in the gel.

Excision of Classical Regions from 7.5% Polyacrylamide Gels

Classical regions 3, 5, and 8, and the interstitial regions were excised from 7.5% PAG. The gels were sliced into the above mentioned regions by comparison with an identical gel stained by the method of Reisner et al. (1975).

The gel sections were triturated, dissolved in sample buffer overnight at 4 C, and then centrifuged at 1144 x g for 20 min. After filtering, the filtrate was collected and applied onto appropriate gels as protein solution.

Densitometric Scanning of Stained Polyacrylamide Gels

A Gilford model 1140 XL gel scanning spectrophotometer was used to scan 17.5% PAG stained with Coomassie Brilliant Blue R at 560 nm.

Phosphoprotein Staining of Polyacrylamide Gels

Phosphoproteins present in the gels were detected by the method of Green, Pastewka, and Peacock (1973). Proteins containing no phosphorus were also stained as a control.

Glycoprotein Staining of Polyacrylamide Gels

Glycoproteins present in the gels were detected by the method of Zacharius, Zell, Morrison, and Woodlock (1969). As a control, gels were subjected to all treatment steps except for the periodic acid treatment.

RESULTS AND DISCUSSION

Electrophoretic Methods

Polyacrylamide Gel Electrophoresis in Single Pore-Size Gels

Figures 3 and 4 demonstrate the electrophoretic patterns obtained in 7.5% PAG in a discontinuous buffer system of proteose-peptone specimens obtained from micellar casein, casein-free centrifuged serum, and skimmilk. The patterns show the classical 3, 5, and 8 zones, with zone 3 notably lacking in the micellar casein specimen. This supports the work of Ng et al. (1970) in that component 3 is not present in the proteose-peptones liberated from washed casein micelles, but is present in large quantities in the serum. This also corroborates the work of Kolar and Brunner (1970) in that components 5 and 8 are casein-associated and are distributed between the casein micelle and the serum.

Region 5 in each specimen appears to be nearly identical except for different concentrations of the zones as evidenced by the intensity of staining. Region 8 appears as an intense, sharp zone moving with the bromophenol blue tracking dye, its mobility unimpeded by the gel matrix. Zones with relative mobilities between those of regions 3 and 5 and between regions 5 and 8 are present in minor concentrations in in each specimen. The casein-free, centrifuged serm specimen showed

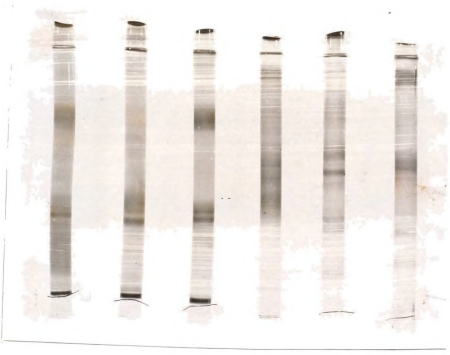


Figure 3. Electrophoretic patterns of proteose-peptone specimens obtained from serum (A), micellar casein (B), and skimmilk (C) electrophoresed in 12.5% (1) and 7.5% (2) PAG.

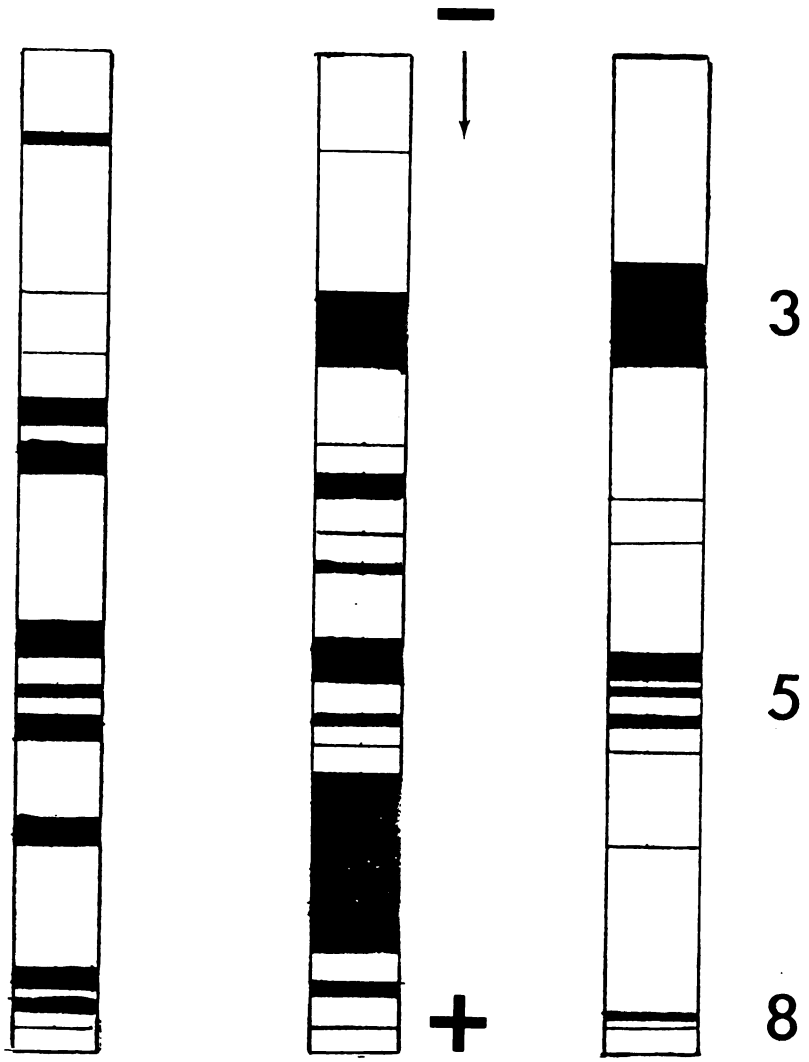


Figure 4. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 7.5% PAG.

profuse zones in the region between 5 and 8. Appearing at the top of the running gel for each specimen is a protein-staining material that did not enter the gel matrix. Apparently a very high molecular weight specie or species is present in the proteose-peptone fraction. Aggregation of smaller molecular weight species is a possible contributory factor to this phenomenon.

When the gel concentration was increased to 10.0% and 12.5%, additional zones were apparent as shown in Figures 3 and 5. Regions 3 and 5 did not migrate as far in these gels, with zone 3 still being the slowest moving zone. More zones, with greater staining intensity, were detected between the 3 and 5 regions and between the 5 and 8 regions. Zones that were moving unimpeded in the 7.5% gels, that is with the tracking dye, appear to have been hindered in their migration by the decreased pore size of the 10.0% and 12.5% gel matrix.

Figures 6 and 7 demonstrate that as gel concentration was further increased to 15.0% and 17.5%, even more zones are evidenced, with as many as 31, 33, and 29 zones being present in the skimmilk, casein-free centrifuged serum, and micellar casein specimens, respectively, in 17.5% PAG. Because of the dramatic resolution obtained as gel concentration was increased, correlations between resulting electrophoretic patterns becomes tenuous. Gel concentrations greater than 17.5% demonstrated fewer zones due to the inability of the slower moving zones to enter the gel. Thus 17.5% gels were used to maximally resolve this heterogeneous protein fraction. However, it must be recognized that by increasing the gel concentration beyond 17.5%, resolution of zones that may be moving together in 17.5% gels even though they differ in size and charge, could be achieved.

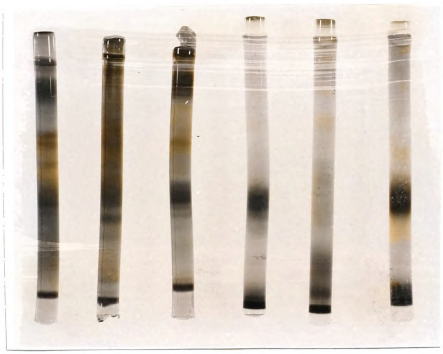


Figure 5. Electrophoretic patterns of proteose-peptone specimens obtained from serum (A), micellar casein (B), and skimmilk (C) electrophoresed in 10% (1) and 5.0% (2) PAG.

Figure 6. Electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 15.0% (1) and 17.5% (2) PAG.

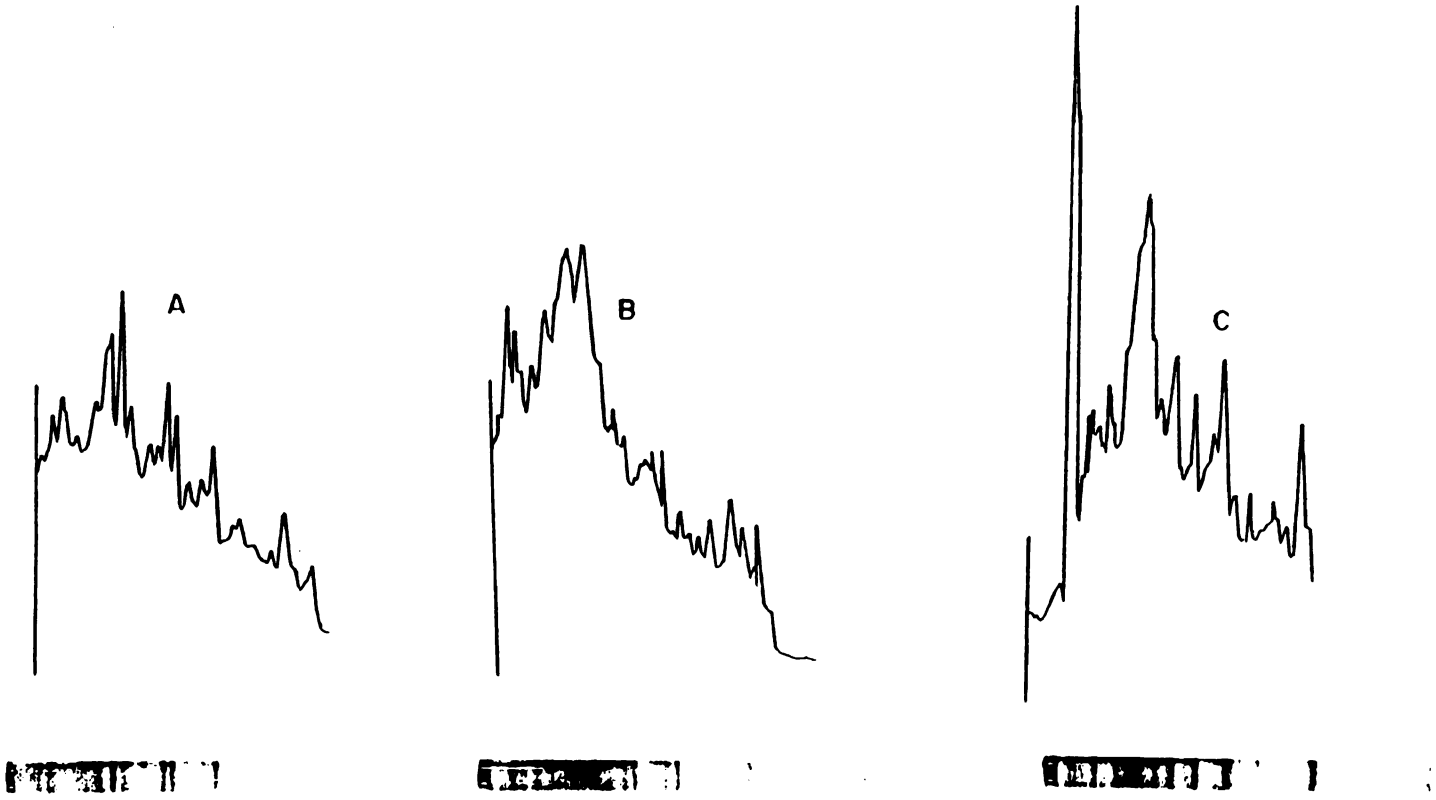


Figure 7. Densitometric scanning patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 17.5% PAG. The corresponding electrophoregrams are beneath the pattern.

Excision of Classical Regions from 7.5% Polyacrylamide Gels and Their Reelectrophoresis in 17.5% Polyacrylamide Gels

The most plausible way to verify from which regions the multiple zones were arising was to excise regions 3, 5, and 8, and the interstitial regions from 7.5% PAG and reelectrophorese them in the resolving 17.5% gels. The proteose-peptone specimen obtained from skim-milk was selected as the protein sample because it represented the classical preparation. The electrophoretic patterns obtained are revealed in Figures 8 and 9.

Excised region 3 accounted for 10 zones in the 17.5% gels with the principal zones being a slow-migrating doublet. The three fastest migrating zones had greater relative mobilities in the 17.5% gels than the corresponding relative mobilities of region 3 in the 7.5% gels. This appears to be contrary to all principles of electrophoretic mobility in polyacrylamide gels. The most specious argument is that some association of the proteins was diminished as the frictional resistance encountered increased while moving through the 17.5% gel matrix. Ng (1967) demonstrated the tendency of component 3 to undergo concentration dependent association-dissociation phenomena. Margolis and Kenrick (1968) found that if "shearing" stress is excessive during gel electrophoresis, loose molecular complexes may be torn apart. Thus it is plausible that component 3 is actually being dissociated by the movement through the 17.5% gel. The law of mass action would increase the tendency for the complex to dissociate further once dissociation commenced.

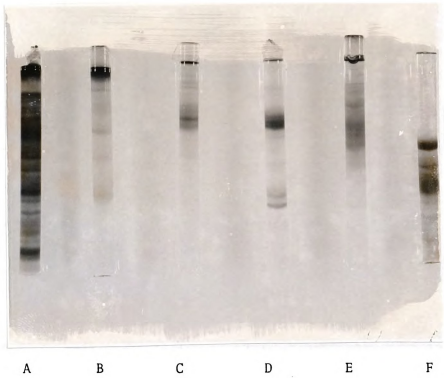


Figure 8. Electrophoretic patterns of proteose-peptone specimens obtained from skimmilk (A), region 3 (B), interstitial region 3→5 (C), region 5 (D), interstitial region 5→8 (E), and region 8 (F) excised from 7.5% PAG and reelectrophoresed in 17.5% PAG.

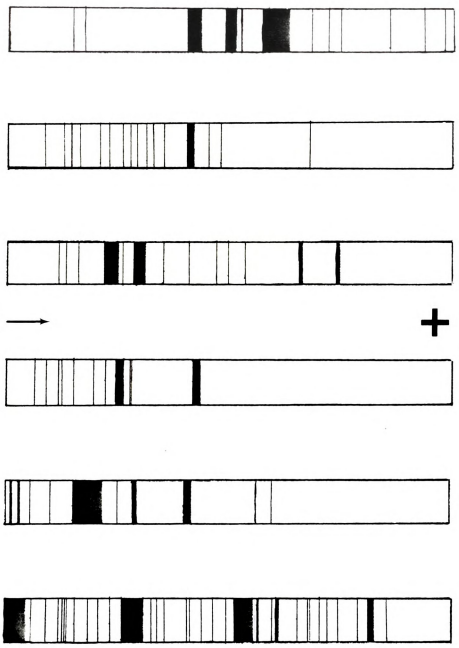


Figure 9. Diagram of electrophoretic patterns of proteose-peptone .Specimen obtained from skimmilk (A), region 3 (B), interstitial region 3→5 (C), region 5 (D), interstitial region 5→8 (E), and region 8 (F) excised from 7.5% PAG and reelectrophoresed in 17.5% PAG.

The excised region between regions 3 and 5 accounted for 10 closely migrating zones and one faster migrating zone when reelectrophoresed in 17.5% PAG.

Excised region 5 appeared as three prominent, closely migrating zones and two major, faster migrating zones. Eight other zones of minor concentration were also apparent. The two fastest migrating zones had very nearly the same relative mobility in 17.5% PAGE as in 7.5% PAGE. This characteristic attests to their low molecular weight.

The excised region lying between regions 5 and 8 appeared as 16 zones in 17.5% PAGE. A minor zone showed an exceptionally fast relative mobility.

Excised region 8 migrated as 12 zones with great dispersity in relative mobilities. Two zones appeared to be greatly hindered in their migration by the high gel concentration. Three zones moved nearly as far as the bromophenol blue tracking dye, evidencing their peptidyl nature.

The proteose-peptone specimen obtained from skimmilk, which was the origin of these 62 zones evidenced from the excised regions, showed only 33 zones when electrophoresed in 17.5% gels. Thus one must realize that the highly resolved electrophoretic patterns obtained from 17.5% PAG underestimates the number of zones actually present. An explanation may be that closely migrating zones may not be distinguishable from each other. The concentration of multiple zones in an area of the gel could result in a dark, wide zone where, indeed, it represents numerous, closely migrating zones.

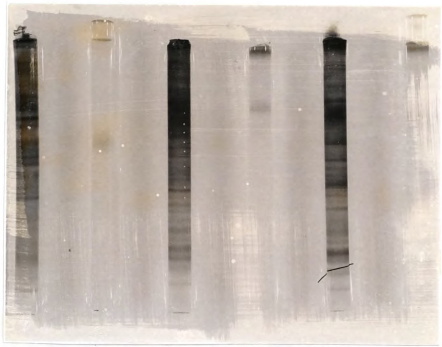
One cannot exclude the possibility that several of the 62 zones evidenced arise from improper slicing of the gels so that overlapping of the excised zones occurred.

Even the 62 zones demonstrated by electrophoresis in the 17.5% gels may underestimate the number of components comprising this fraction. Genetic variants which differ in histidine residues may move together in alkaline PAGE, but could be separated in acidic PAGE. Peterson and Kopfler (1966) demonstrated this for the genetic variants of β-casein A. Also one must realize that the proteinaceous material which did not enter the gel matrix could account for additional zones.

Differential Staining of 17.5% Polyacrylamide Gels for Phosphorus and for Carbohydrate

The results from staining 17.5% gels for carbohydrate and for phosphorus are represented in Figures 10 and 11. Figure 12 indicates, utilizing the results of the staining procedure, those zones which are classified as proteins, phosphoproteins, glycoproteins, and phosphoglycoproteins. The two slow migrating zones of region 3 stained for both phosphorus and carbohydrate, verifying the work of Ng *et al.* (1970) in which they found component 3 to be a phosphoglycoprotein.

Three prominent zones, which can be attributed to region 5, stained for phosphorus in the proteose-peptone specimens obtained from skimmilk and casein-free, centrifuged serum. In each of these specimens one of the zones stained for carbohydrate, but these zones did not appear to have the same relative mobilities. The proteose-peptone specimen obtained from micellar casein demonstrated six zones in the 5 region, with three containing phosphorus; carbohydrate was not detected. In proteose-peptone specimens obtained from both casein-free, centrifuged



1A 2A 1B 2B 1C 2C

Figure 10. Electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 17.5% PAG stained for protein (1) and for carbohydrate (2).

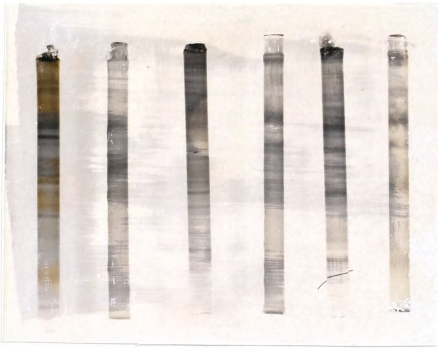


Figure 11. Electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 17.5% PAG stained for protein (1) and for phosphorus (2).

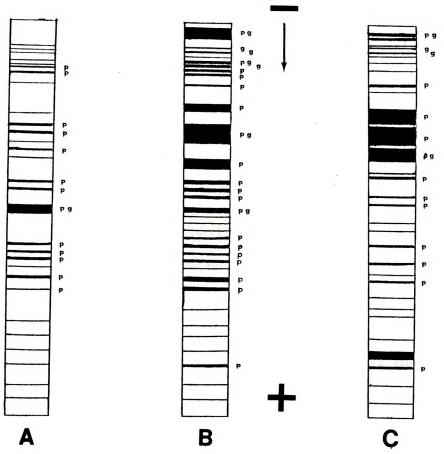


Figure 12. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 17.5% PAG demonstrating those zones which are proteins, phosphoproteins (p), glycoproteins (g), and phosphoglycoproteins (pg).

serum and micellar casein, a phosphoglycoprotein was detected with a relative mobility greater than either of the phosphoglycoproteins found in the 5 region.

The proteose-peptone specimens obtained from both casein-free, centrifuged serum and skimmilk showed glycoproteins moving slightly ahead of the two primary zones of region 3. One of these zones from the serum specimen appeared to be a phosphoglycoprotein.

The fast migrating zones in 17.5% gels appear to be void of phosphorus. Such swift moving zones, without deriving a great share of their mobility from phosphorylated residues, must be very acidic and/or very small in size.

The small pore size of the 17.5% gels would indicate that the separation achieved is based more on size than on charge. The electrophoretic pattern obtained is a fairly representative reflection of the molecular weight of the proteins in the zones--higher molecular weight species at the top of the gel and smaller species nearer the anodic end of the gel. This is not to infer that a larger, highly charged protein cannot migrate farther than a smaller protein possessing a lower charge. Electrophoretic resolution on polyacrylamide gels still leaves open the question of whether two proteins are separated because they differ in charge and/or size.

Polyacrylamide Gel Electrophoresis in 10-25% and 15-30% Gradient Pore Gels

Electrophoretic patterns obtained when the three proteose-peptone specimens were electrophoresed in 10-25% and 15-30% linear gradient gels are represented in Figure 13. Proteose-peptone specimens obtained from skimmilk, casein-free, centrifuged serum, and micellar

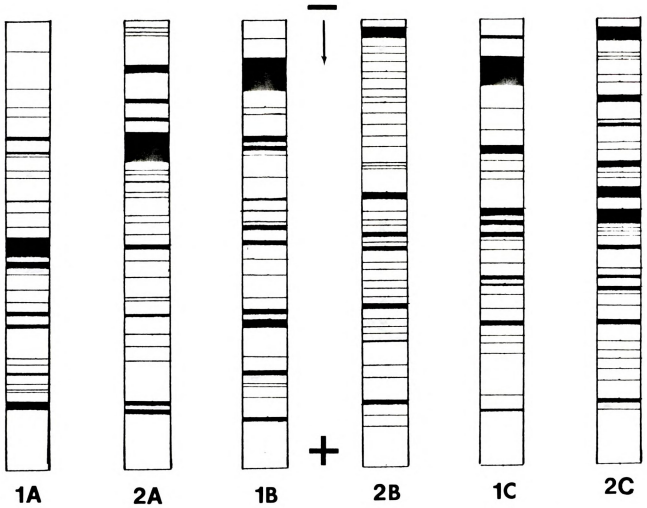


Figure 13. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 10-25% linear gradient PAG (1) and 15-30% linear gradient PAG (2).

casein demonstrated 24, 24, and 26 zones, respectively, in 10-25% polyacrylamide linear gradient gels and 33, 38, and 26 zones, respectively, in 15-30% polyacrylamide linear gradient gels.

The rationale for employing gels consisting of decreasing pore size was to decrease the migration rate of the proteins, so that a particular protein would reach its pore limit, and very little alteration in zonal pattern would result with further passage of current (Margolis and Kenrick, 1968). This effect was not achieved totally since the leading zones were still moving with the ion front at the end of electrophoresis, obviously being unimpeded by the gel matrix.

Rodbard, Kapadia, and Chrambach (1971) dispute the idea of "dead-stop electrophoresis" as proposed by Margolis and Kenrick (1968). They maintained that a limiting pore size cannot be attained and that gradient gels are, at best, useful for initial "mapping" of complex mixtures. Because of the large variation of pore size in polyacrylamide gels at any given concentration, increased resolution cannot be obtained.

Polyacrylamide Gel Electrophoresis Containing Sodium Dodecyl Sulfate

The proteose-peptone specimens obtained from skimmilk, casein-free, centrifuged serum, and micellar casein revealed 12, 17, and 15 zones, respectively, of differing molecular weights. Twenty-two different molecular weight species were found collectively in the specimens, ranging in molecular weight from <3000 to ~90,000, as shown in Table 2. Different gel concentrations were required to resolve these diverse molecular weight species because a single gel concentration could not separate adequately both large proteins and small peptides. (See Figures 14-23.) High molecular weight components (>38,000) were

Table 2.--Molecular weights of proteose-peptone specimens determined from 5.0%, 7.5%, 10.0%, 12.5% and 17.5% SDS-PAGE.

Skimmilk	Micellar Casein	Serum
	90,000 ± 2000[a,b]	
	80,000 ± 2000[a,b,c]	
	74,000 ± 2000[a,b,c]	74,000 ± 2000[a,b,c]
68,000 ± 1000[b,c]	69,000 ± 1000[b,c]	71,000 ± 2000[b,c]
	66,000 ± 1000[b,c]	66,000 ± 1000[b,c]
61,000 ± 1000[b,c,d]	61,000 ± 1000[b,c,d]	61,000 ± 1000[b,c,d]
55,000 ± 1000[c,d]	56,000 ± 1000[c,d]	55,000 ± 1000[c,d]
51,000 ± 1000[c,d]		51,000 ± 1000[b,c,d]
44,000 ± 1000[c,d]		44,000 ± 1000[b,c,d]
37,000 ± 1000[c,d,e]	37,000 ± 1000[c,d,e]	38,000 ± 1000[c,d,e]
	31,000 ± 1000[d,e]	
		28,000 ± 1000[d,e]
25,000 ± 1000[d,e]	24,000 ± 1000[d,e]	25,000 ± 1000[d,e]
	22,000 ± 1000[d,e]	23,000 ± 1000[d,e]
20,000 ± 1000[d,e]		19,000 ± 1000[d,e]
14,000 ± 1000[d,e]		14,000 ± 1000[d,e]
	12,000 ± 1000[d,e]	11,000 ± 1000[d,e]
8,200 ± 300[e]	8,200 ± 300[e]	
7,200 ± 300[e]		7,200 ± 300[e]
		5,700[e]
	4,600[e]	
<3,000[e]	<3,000[e]	<3,000[e]

[a]determined from 5.0% SDS-PAGE; [b]determined from 7.5% SDS-PAGE; [c]determined from 10.0% SDS-PAGE; [d]determined from 12.5% SDS-PAGE; [e]determined from 17.5% SDS-PAGE.

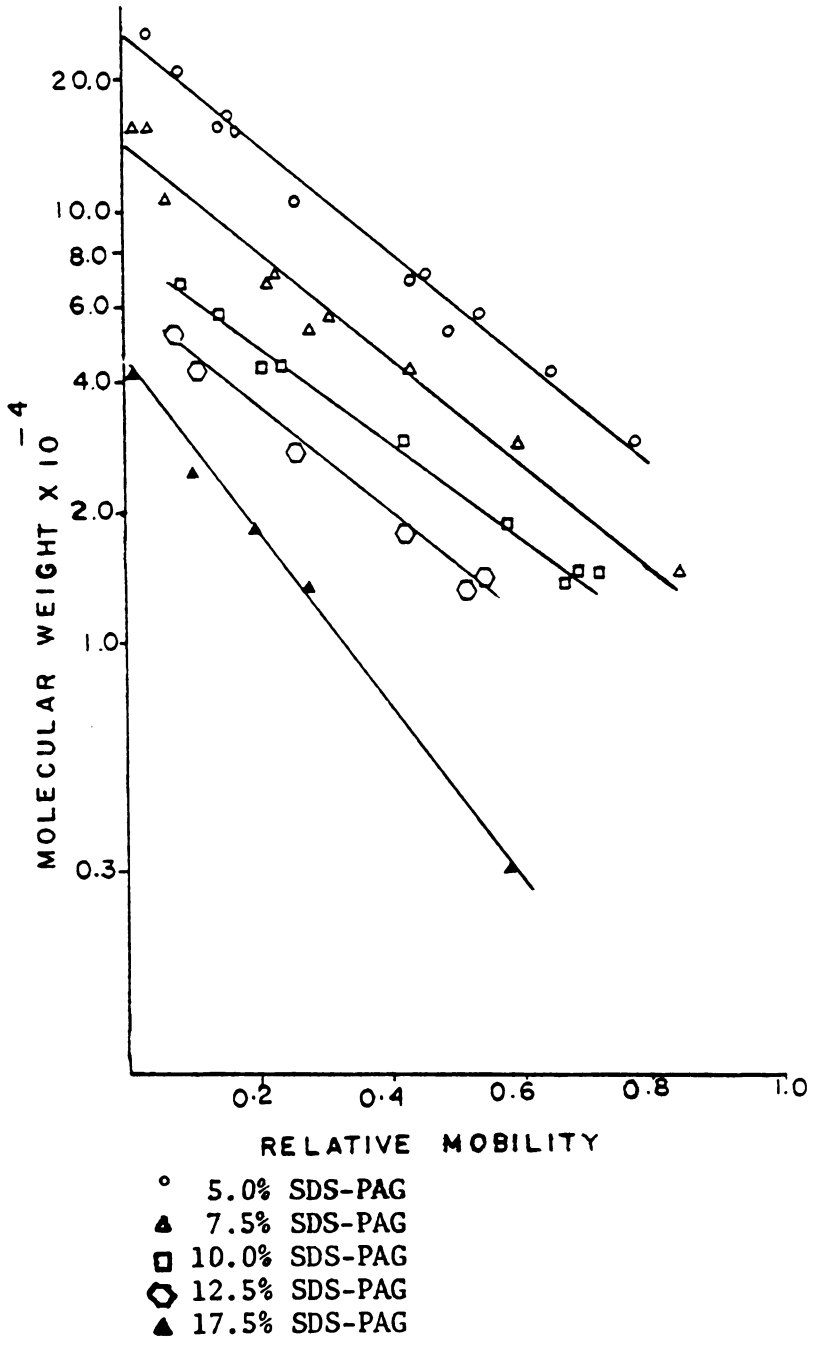


Figure 14. Standard curve for molecular weight determination in 5.0%, 7.5%, 10.0%, 12.5%, and 17.5% SDS-PAG.

A B C D E F

Figure 15. Electrophoretic patterns of BDH standard proteins, 53,000-265,000 (B), 14,300-71,500 (C), RNA polymerase (E. coli) (A), bovine serum albumin (A), and proteose-peptone specimens obtained from micellar casein (F), serum (E), and skimmilk (D) electrophoresed in 5.0% SDS-PAG.

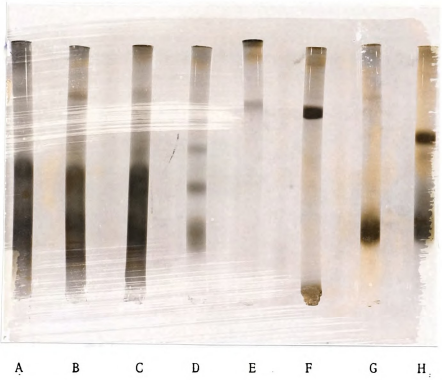
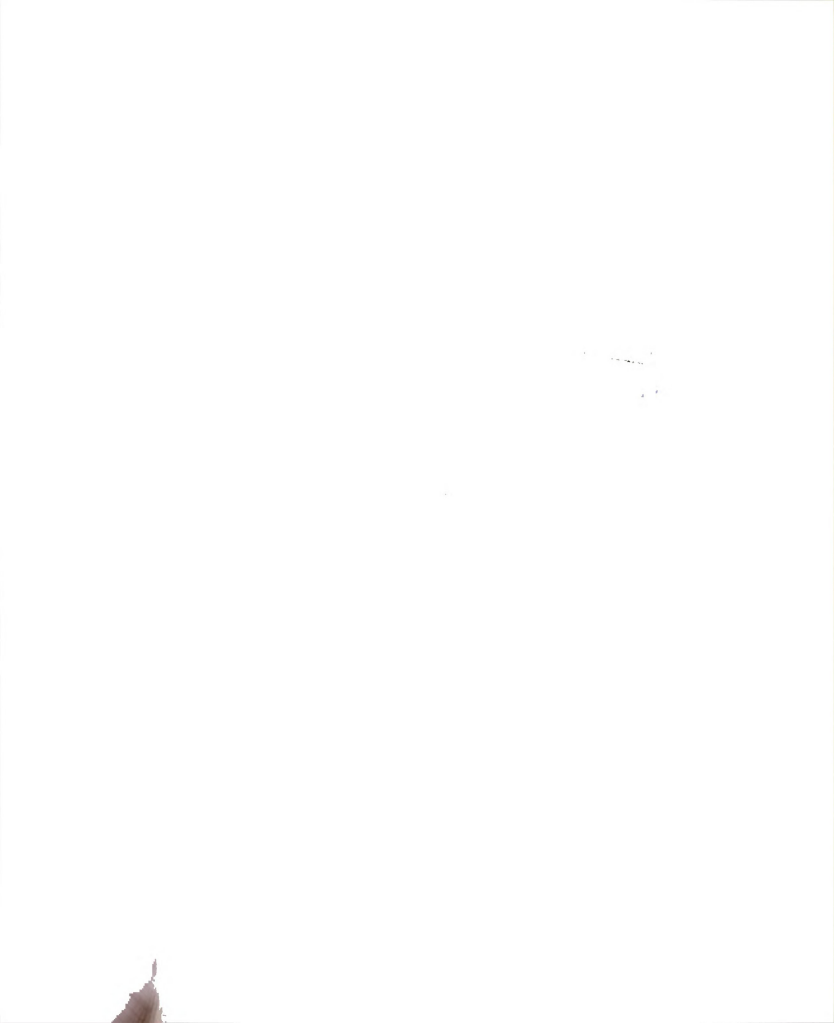


Figure 16. Electrophoretic patterns of BDH standard proteins, 53,000-265,000 (E), 14,300-71,500 (D), bovine serum albumin (F), ribonuclease (G), ovalbumin (H), β-lactoglobulin B (H), and proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 7.5% SDS-PAG.



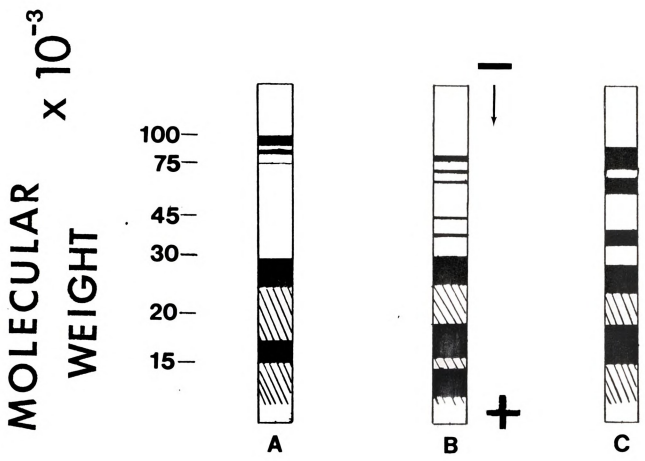


Figure 17. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 7.5% SDS-PAG.

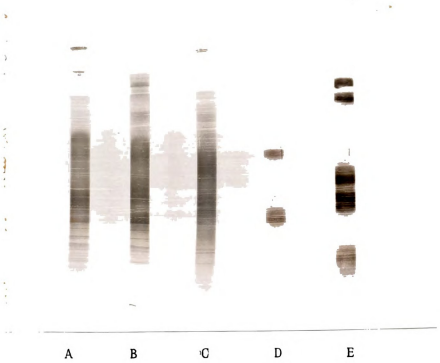


Figure 18. Electrophoretic patterns of BDH standard proteins, 14,300-71,500 (D), L-glutamic dehydrogenase (E), ovalbumin (E), ß-lactoglobulin B (E), ribonuclease (E), insulin (E), and proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 10.0% SDS-PAG.

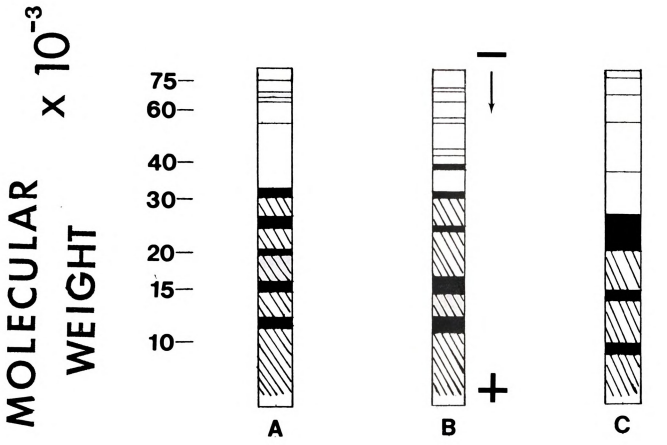


Figure 19. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 10.0% SDS-PAG.

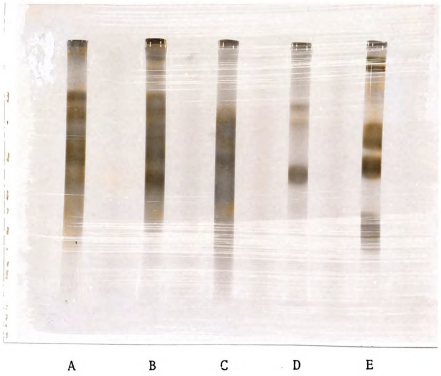


Figure 20. Electrophoretic patterns of BDH standard proteins, 14,300-71,500 (D), L-glutamic dehydrogenase (E), ovalbumin (E), ß-lactoglobulin B (E), ribonuclease (E), insulin (E), and proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 12.5% SDS-PAG.

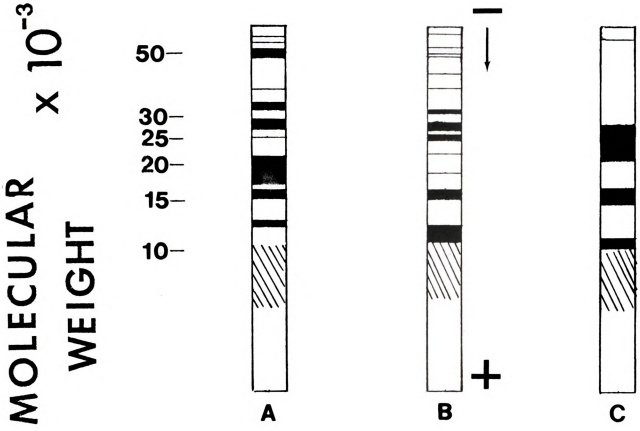


Figure 21. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 12.5% SDS-PAG.

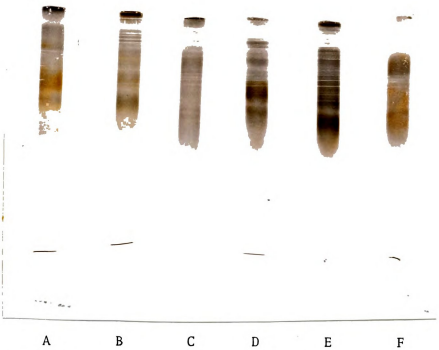


Figure 22. Electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 17.5% SDS-PAG.

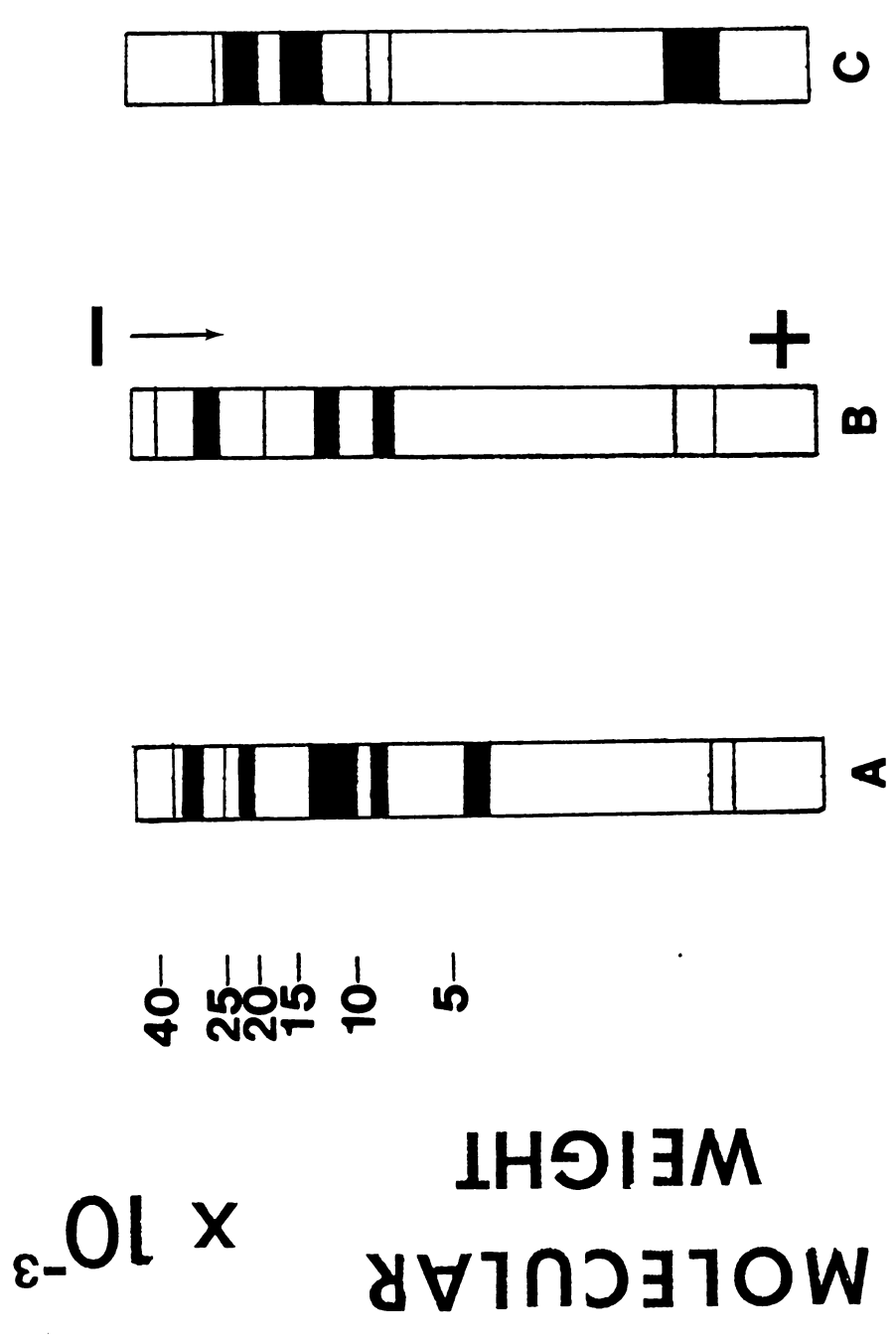


Figure 23. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in 17.5% SDS-PAG.

estimated from 5.0%, 7.5%, and 10.0% SDS-PAGE, while molecular weight species of $\leq$38,000 were determined from 12.5% and 17.5% SDS-PAGE. To separate peptides of $\leq$8200 daltons, electrophoresis in 17.5% gels was required.

Segrest and Jackson (1972) have shown that glycoproteins behave anomalously in SDS-PAGE. Evidently glycoproteins bind less SDS than simple proteins. Thus their molecular weight is often overestimated because of a slower rate of migration. However, by increasing gel concentration, molecular sieving predominates and the anomalously high apparent molecular weight of glycoproteins decreases, approaching in an asymptotic manner, values close to their real molecular weights.

The proteose-peptone fraction contains glycoproteins; thus the five gel concentrations employed in this study would be advantageous in detecting anomalous electrophoretic behavior of the protein components. The results of this study indicated that no anomalous behavior was apparent.

The presence of molecular weights of components >38,000 is surprising, in that previously no completely dissociated species with molecular weights of >40,000 have been demonstrated to be present in this fraction. Ng (1967) found component 3 to have a molecular weight of 200,000 at infinite dilution in veronal buffer in the ultracentrifuge. This component showed concentration-dependent association phenomena. In 5M guanidine hydrochloride component 3 demonstrated a molecular weight of 40,000. Several milk proteins have been shown to have molecular weights >40,000, but they have not been demonstrated to be stable to heating at 95 C for 40 min. Possibly the proteose-peptone fraction contains components that are rendered heat-stable by

glycosylation. A thorough carbohydrate staining of the electrophoretic zones in SDS-PAGE would document this. It is also plausible that associated smaller molecular weight species have not been dissociated by the SDS and heat treatment employed in preparing the protein sample for electrophoresis. However, the SDS treatment used in this study was typical of the manner in which proteins are treated prior to SDS-PAGE. Also, the 1% 2-mercaptoethanol used should have reduced any existing disulfide bonds.

The zones of molecular weight $\leq$31,000 appeared as diffuse zones extending from the 31,000 dalton region to the region immediately behind the marker dye. Gels prepared with increasingly higher concentrations of polyacrylamide were required to resolve closely migrating zones in this region. Zones differing in molecular weight of ~1000-2000 could be distinguished from each other in 12.5% and 17.5% SDS-PAG.

The preponderance of zones at molecular weights of $\leq$31,000 should be noted because they are in the molecular weight region of the caseins. Possibly some of the minor glycosylated caseins in milk survive acid precipitation and are recovered in the proteose-peptone fraction. Also the possibility exists that zones assigned the molecular weights of 31,000, 28,000, 25,000, 24,000, 23,000, 20,000, and 19,000 represent associated species of cleavage products of major casein components.

The molecular weight species in the 14,000, 12,000, and 8200 regions are quite similar to the 14,300 and 9900 dalton species of component 5 and 8-slow, respectively, reported by Kolar and Brunner (1970).

By extrapolation from the standard curve (Figure 14), the molecular weight of the zones near the bromophenol blue tracking dye were estimated at <1000. Actually, the only conclusion that can be drawn is that the molecular weights are less than that of insulin.

Excision of Classical Regions from 7.5% Polyacrylamide Gels and Their Reelectrophoresis in 17.5% Sodium Dodecyl Sulfate Polyacrylamide Gels

To better assess the molecular weights of the classical 3, 5, and 8 regions, a proteose-peptone specimen from skimmilk was electrophoresed first in 7.5% PAG. Regions of this gel containing the classical 3, 5, and 8 components, as well as the interstitial regions were excised and reelectrophoresed in 17.5% SDS-PAG (see Figures 24 and 25).

Excised region 3 showed a major zone of 37,000 daltons and minor zones of 31,000, 24,000, and 15,000 daltons (see Table 3). The 37,000 dalton zone correlates well with the molecular weight of component 3 found by Ng (1970) in 5M guanidine hydrochloride in the ultracentrifuge (~40,000). The minor zones could be smaller proteins that are associated with component 3.

The excised region between regions 3 and 5 showed molecular species of 37,000, 30,000, 18,000, and 14,000 daltons, and a minor zone at 24,000 daltons.

Excised region 5 contained a prominent zone of molecular weight 26,000, with minor zones of 14,000 and 12,000 daltons.

The excised region between regions 5 and 8 demonstrated a lone zone of 25,000 daltons.

Excised region 8 showed two closely migrating zones with mobilities extending beyond that of the lowest molecular weight standard

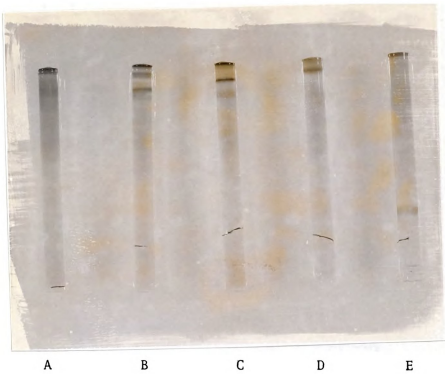


Figure 24. Electrophoretic patterns of region 3 (A), interstitial region 3↔5 (B), region 5 (C), interstitial region 5↔8 (D), and region 8 (E) excised from 7.5% PAG and reelectrophoresed in 17.5% SDS-PAG.

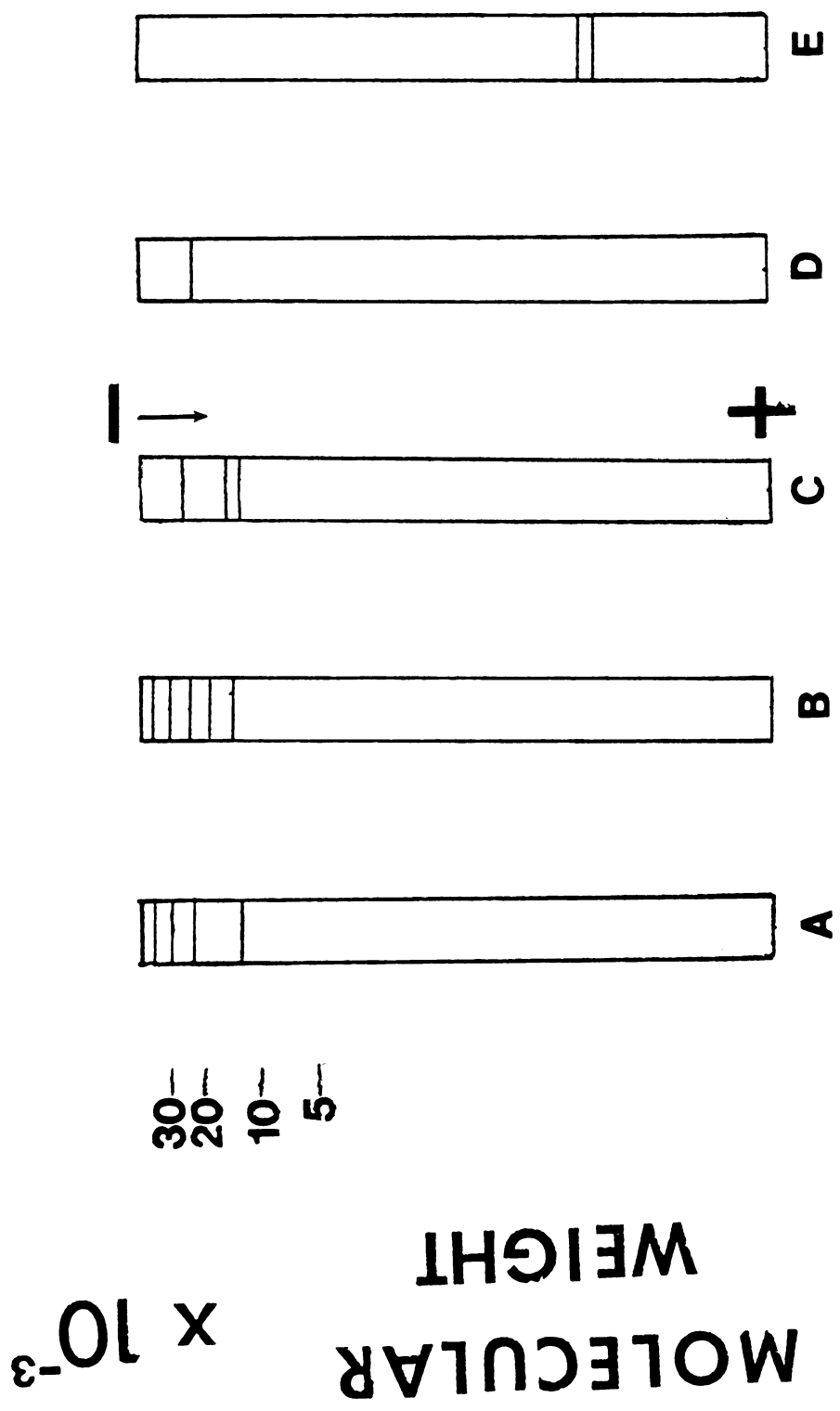


Figure 25. Diagram of electrophoretic patterns of region 3 (A), interstitial region 3→5 (B), region 5 (C), interstitial region 5→8 (D), and region 8 (E) excised from 7.5% PAG and reelectrophoresed in 17.5% SDS-PAG.

Table 3.--Molecular weights of regions 3, 3→5, 5, 5→8, and 8 excised from 7.5% PAG determined from 17.5% SDS-PAG.[a]

Region 3	Region 3→5	Region 5	Region 5→8	Region 8
37,000	37,000	26,000	25,000	<3000
31,000	30,000	14,000		
24,000	24,000	12,000		
15,000	18,000			
	14,000			

[a]Average of two determinations.

used (insulin) with molecular weights estimated at <1000 by extrapolation of the standard curve. This data reestablishes the possibility that region 8 contains components of peptidyl nature.

The molecular weights obtained in this study are not to be interpreted as unequivocal values. The only true criterion of molecular weight is the amino acid sequence of homogeneous components. Anomalous results have been reported in SDS-PAGE. Different proteins do not bind the same amount of SDS (Nelson, 1972). Anomalous binding of SDS by proteins, atypical conformation of the protein-SDS complex, or unusual properties of the native protein-SDS complex maintained in a SDS solution can affect the mobility of proteins (Banker and Cofman, 1972). Neville (1971) found that components of molecular weight <15,000 are out of the linear region of the standard equation employed to determine the mobility of macromolecules through the gel matrix. Williams and Gratzer (1971) showed that SDS complexes formed by all proteins of molecular weight <6000 migrate with the same mobility. This behavior is believed to occur because the frictional coefficients of SDS complexes of this size are no longer dependent on their molecular weight. Svasti and Panijpan (1977) explain this anomalous behavior from the fact that the dimensions of the minor and major axes of the SDS-protein complex are not sufficiently different, so that their hydrodynamic behavior approaches that of a sphere instead of a cylinder, as is common for larger proteins. Swank and Munkres (1971) stated that the anomalous behavior of small peptides in SDS-PAGE is due to three reasons: (1) the intrinsic charge of the peptides becomes more important at lower molecular weights, (2) the shape of the peptide-SDS complex could cause it to move through the gel matrix in a manner that is

not a function of its molecular weight, (3) differential binding of SDS by different protein species, that is, SDS may bind more to hydrophobic peptides, and less to hydrophilic peptides.

The above reasons for anomalous behavior of peptides during SDS-PAGE could assist in explaining the results of SDS-PAGE with that of conventional PAGE for region 8. Region 8 may consist of many differing sized species distributed in the peptide region. Thus region 8 proteins which show only two zones by SDS-PAGE separate into 12 zones by PAGE.

Isoelectric Focusing in Polyacrylamide Gel

Isoelectric focusing in polyacrylamide gel, employing pH 3.0-5.0 and pH 4.0-6.0 ampholytes, demonstrated that a linear gradient was produced (see Figure 26), and that the proteose-peptone fraction consisted of components in the pI 3.8 to 5.3 region. The proteose-peptone specimens obtained from micellar casein, serum, and skimmilk showed 23, 20, and 13 zones, respectively in IEF-PAG (see Figure 27). Most of the zones were found in the pI 4.3 to 4.8 region.

More zones may have been detected if the pH gradient covered a narrower range.

Closely migrating zones were difficult to distinguish due to background staining. The method of Reisner et al. (1975) was found to be the best method of staining to prevent the ampholytes from staining. However, some background staining was observed.

Some zones precipitated upon reaching their isoelectric point. This was apparent by a white precipitation band which developed. Electrofocusing was continued after the precipitation bands appeared

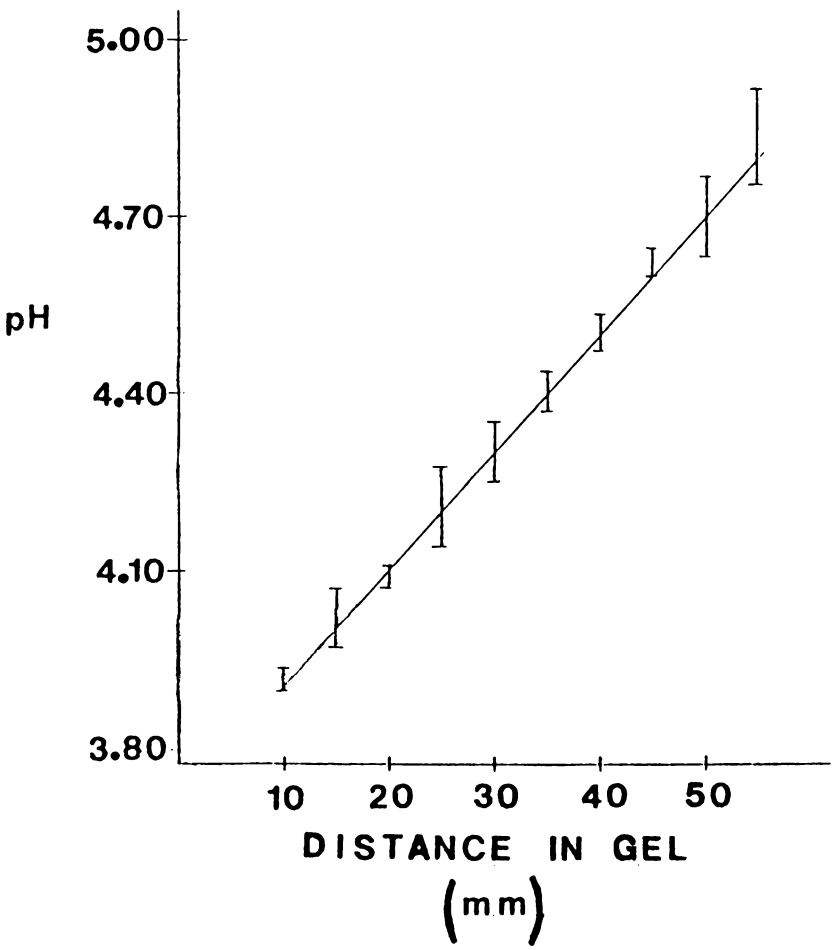


Figure 26. Standard curve of pH 3-6 linear gradient in IEF-PAG.

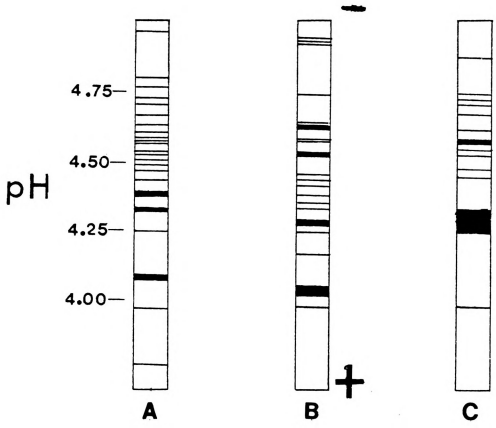


Figure 27. Diagram of electrophoretic patterns of proteose-peptone specimens obtained from micellar casein (A), serum (B), and skimmilk (C) electrophoresed in pH 3-6 linear gradient IEF-PAG.

to insure that other proteins had sufficient time to reach their isoelectric zones. Because the pH gradient undergoes a progressive flattening with time of electrofocusing, called the "plateau phenomenon" (Finlayson and Chrambach, 1971), it would be unreliable to determine isoelectric points of precipitated proteins from pH measurements long after they had precipitated. Thus, all that can be ascertained is that the proteose-peptone fraction consists of components in the pI 3.8 to 5.3 region.

The isoelectric points of the caseins are in this isoelectric pH region. Possibly some of the proteose-peptones are caseins or consist of casein segments derived from the caseins.

There is the possibility in IEF-PAG that two proteins may have the same isoelectric point, but actually be different in molecular size. Such proteins would be indistinguishable, appearing as one zone in the electrophoretic pattern. The possibility that this phenomenon was operative in this study is reinforced by the consideration that the proteose-peptone fraction consists, in part, of proteolytic breakdown products of other milk proteins. Breakdown products derived from the same region of the native protein, but with several more or less uncharged amino acids, would be inseparable in IEF-PAG but separable by 17.5% PAG. This could account for the better resolution by PAGE then IEF-PAG.

Origin of the Proteose-Peptone Fraction

The proteose-peptone fraction of cow's milk derives its name from the supposition that its components are heat-derived breakdown products of major milk proteins, consisting of proteoses which are

precipitable by one-half saturated ammonium sulfate, and of peptones which are precipitable by saturated ammonium sulfate. Since several workers (Larson and Rolleri, 1955; Kolar and Brunner, 1970; Ng et al., 1970) have demonstrated the presence of proteose-peptone components in unheated skimmilk, the above mentioned supposition can be refuted.

With the increasing importance being ascribed to the role of intrinsic milk protease (plasmin) in the milk protein system (Kaminogawa and Yamauchi, 1972; Kaminogawa, Mizobuchi, and Yamauchi, 1972; Yamauchi and Kaminogawa, 1972; Groves, Gordon, Kalan, and Jones, 1973; Eigel, 1977a,b), one can postulate that this trypsin-like enzyme found in milk could be a primary source of the proteose-peptone components. Groves et al. (1973) isolated the gamma caseins from skimmilk, demonstrating that they are indigenous to milk, and are actually the 106-209 and 108-209 segments of β-casein A^2. Eigel (1977a,b) showed that plasmin is responsible for gamma casein formation from β-casein A^2.

The 1-105 and 1-107 N-terminal segments of β-casein have not been discovered in the milk protein system. Could not these highly acidic, phosphorylated peptides be present in the proteose-peptone fraction? One worker has already made this hypothesis (Jenness, 1978). By comparing the amino acid composition of component 5 of the proteose-peptone fraction, as isolated by Kolar and Brunner (1970), with that of the known amino acid sequence of the 1-107 peptide of β-casein A^2, Jenness uncovered an uncanny correlation in amino acid residues and in phosphorus content. This comparison is shown in Table 4.

Since Kolar's component 5 was not pure, the presence of 1.5% carbohydrate (which is minute) could result from a contaminating glycoprotein or phosphoglycoprotein. In the author's study the 5

Table 4.--Comparison of amino acid compositions of peptide 1-107 of β-casein A^2 and component 5.[a]

Residue	Peptide 1-107 of β-casein A^2			Component 5[b]	
	No. per mole	Wt. per residue	Wt. per mole	g/100g	moles / 12,469
Lys	8	128	1024	7.55	7.4
His	2	137	274	1.92	1.8
Arg	2	156	312	2.60	2.1
Asp	6	115	690	5.81	6.3
Thr	5	101	505	4.72	5.8
Ser	9	87	783	5.77	8.3
Glu	24	129	3096	24.89	24.0
Pro	15	97	1455	10.55	13.6
Gly	2	57	114	1.23	2.7
Ala	3	71	213	1.92	3.4
1/2 Cys	-	-	-	-	-
Val	9	99	891	6.60	8.3
Met	2	131	262	1.68	1.6
Ile	7	113	791	6.14	6.8
Leu	8	113	904	7.33	8.1
Tyr	1	163	163	1.94	1.5
Phe	4	147	588	4.86	4.1
Trp	-	-	-	-	-
H_2PO_3	5	81	405	2.53	3.9
Correction[c]			-1		
Total	107		12,469		

[a]From Jenness (1978).

[b]Data from Kolar and Brunner (1970).

[c]Add 18 for N-terminal H and C-terminal OH, subtract 14 for difference between weights of $-NH_2$ and -OH for 14 amides, subtract 5 for H's lost from serine by esterification with H_2PO_3.

region did demonstrate the presence of a phosphoglycoprotein. Concentration also plays an important role in the amino acid data. Minor impurities in Kolar's preparations would not contribute significantly to the data. Also assuming the impurities in component 5 to be caseins, or casein-derived, less deviation could occur because of the quite similar amino acid compositions of each of the caseins. Thus Kolar and Brunner may have indeed isolated the 1-107 and/or 1-105 segments of β-casein A^2 which result from the action of milk protease (plasmin) on β-casein A^2. Moreover, some of the zones in the 5 region evidenced in this study appear to represent these two peptides. Possibly the genetic variants ascribed to the N-terminal segments of β-casein are also present in the 5 region. This is reinforced by the molecular weights of 14,000 and 12,000 found for the excised 5 region by SDS-PAGE.

The possibility that segment 1-28 of β-casein, resulting from the formation of γ_1-A^1, γ_1-A^2, γ_1-A^3, and γ_1-B caseins from β-casein by the action of milk protease, or plasmin, are present in the 8-fast fraction, as isolated by Kolar and Brunner (1970), is plausible. A good correlation results when the amino acid composition of Kolar's 8-fast is compared to the known sequence of segment 1-28 of β-casein, as represented in Tables 5 and 6.

Data from the unheated specimen shows a better correlation between the residues of phosphorus of component 8-fast with that of the 1-28 segment than does data from the heated specimen. Perhaps heating of the skimmilk in the first step of the preparation of the specimen may have cleaved some of the phosphorus from the serine monophosphoesters. Since Kolar and Brunner's component 8-fast was not pure, the same rationale can be used as that of component 5--contaminating

Table 5.--Comparison of amino acid composition of peptide 1-28 of β-casein A[2] and component 8-fast (unheated).

Residue	Peptide 1-28 of β-casein A[2]			Component 8-fast[a] (unheated)	
	No. per mole	Wt. per residue	Wt. per mole	g/100g	moles / 3472
Lys	1	128	128	7.92	2.1
His	-	-	-	-	-
Arg	2	156	312	3.63	0.8
Asp	2	115	230	8.20	2.5
Thr	1	101	101	4.48	1.5
Ser	5	87	435	12.17	4.9
Glu	7	129	903	23.96	6.4
Pro	1	97	97	2.51	0.9
Gly	1	57	57	1.21	0.7
Ala	-	-	-	.80	0.5
1/2 Cys	-	-	-	-	-
Val	2	99	198	5.57	2.0
Met	-	-	-	-	-
Ile	3	113	339	8.41	2.6
Leu	3	113	339	4.27	1.3
Tyr	-	-	-	1.45	0.3
Phe	-	-	-	.73	0.2
Trp	-	-	-	.06	-
H_2PO_3	4	81	324	8.59	3.7
Correction[b]			+9		
Total	28		3472		

[a]Data from Kolar and Brunner (1970).

[b]Add 18 for N-terminal H and C-terminal OH, subtract 5 for difference between weights of $-NH_2$ and -OH for 5 amides, subtract 4 for H's lost from serine by esterification with H_2PO_3.

Table 6.--Comparison of amino acid compositions of peptide 1-28 of β-casein A[2] and component 8-fast (heated).

Residue	Peptide 1-28 of β-casein A[2]			Component 8-fast[a] (heated)	
	No. per mole	Wt. per residue	Wt. per mole	g/100g	moles / 3472
Lys	1	128	128	5.22	1.4
His	-	-	-	.82	0.2
Arg	2	156	312	5.42	1.2
Asp	2	115	230	8.11	2.4
Thr	1	101	101	5.04	1.7
Ser	5	87	435	10.00	4.0
Glu	7	129	903	25.30	6.8
Pro	1	97	97	3.67	1.3
Gly	1	57	57	1.19	0.7
Ala	-	-	-	.93	0.6
1/2 Cys	-	-	-	-	-
Val	2	99	198	5.18	1.8
Met	-	-	-	1.10	0.3
Ile	3	113	339	7.36	2.3
Leu	3	113	339	7.16	2.2
Tyr	-	-	-	.70	0.1
Phe	-	-	-	1.04	0.2
Trp	-	-	-	.23	-
H_2PO_3	4	81	324	6.26	2.7
Correction[b]			+9		
Total	28		3472		

[a]Data from Kolar and Brunner (1970).

[b]Add 18 for N-terminal H and C-terminal OH, subtract 5 for difference between weights of $-NH_2$ and -OH for 5 amides, subtract 4 for H's lost from serine by esterification with H_2PO_3.

species account for the small discrepancies in amino acid data and in the presence of the ~2% carbohydrate.

Possibly one of the phosphorus staining zones migrating near the tracking dye in this study represents this highly charged peptide.

Kolar and Brunner (1970) isolated proteose-peptone component 8-slow. Its molecular weight was determined to be 9900 in the ultracentrifuge. This might represent the 29-105 and 29-107 segments of β-casein which would result if cleavage of the 1-105 and 1-107 segments occurred at the Lys-Lys bond as demonstrated in the formation of γ_1-A^1, γ_1-A^2, γ_1-A^3, and γ_1-B caseins. However, this does not appear to be the case. A comparison of the amino acid and phosphorus data of Kolar and Brunner with that of the known sequence of this segment of β-casein A^2 indicates a poor correlation (see Table 7). The high carbohydrate content present in 8-slow also supports the argument that 8-slow does not represent the 29-105 and 29-107 segments of β-casein A^2.

However, the possibility that component 8-slow could be derived from the other caseins is a distinct possibility. Its high glutamic acid and aspartic acid contents, along with a phosphorus content of 5.8% and a low proline content, suggests that it is derived from the hydrophilic region of α_s-casein. Possibly milk protease, or plasmin, cleaves α_s-casein in a trypsin-like manner analogous to its action on β-casein.

Eigel (1977b) demonstrated (in vitro) that α_s-casein is broken down by plasmin, but not as readily as β-casein. He found the predominant breakdown species to have molecular weights at 20,500, 12,300, and 10,300.

Table 7.--Comparison of amino acid composition of peptide 29-107 of β-casein A[2] and component 8-slow (unheated).

Residue	Peptide 29-107 of β-casein A[2]			Component 8-slow[a] (unheated)	
	No. per mole	Wt. per residue	Wt. per mole	g/100g	moles 9015
Lys	7	128	896	8.79	6.2
His	2	137	274	5.63	3.7
Arg	-	-	-	1.36	0.8
Asp	4	115	460	15.34	12.0
Thr	4	101	404	4.49	4.0
Ser	4	87	348	8.67	9.0
Glu	17	129	2193	15.40	10.8
Pro	14	97	1358	3.38	3.1
Gly	1	57	57	.53	0.8
Ala	3	71	213	2.13	2.7
1/2 Cys	-	-	-	-	-
Val	7	99	693	2.40	2.2
Met	2	131	262	.67	0.5
Ile	4	113	452	2.90	2.3
Leu	5	113	565	6.27	5.0
Tyr	1	163	163	.68	0.4
Phe	4	147	588	2.42	1.5
Trp	-	-	-	.23	0.1
H_2PO_3	1	81	81	5.79	6.4
Correction[b]			+8		
Total	79		9015		

[a]Data from Kolar and Brunner (1970).

[b]Add 18 for N-terminal H and C-terminal OH, subtract 9 for difference between weights of $-NH_2$ and -OH for 9 amides, subtract 1 for H lost from serine by esterification with H_2PO_3.

Reimerdes and Klostermeyer (1974) demonstrated that milk protease is associated with casein micelles. Milk protease, with its ready access to the caseins, could cleave susceptible casein substrates, forming proteose-peptone components. These components could be held in the calcium-apatite complex, being released upon acidification. Kolar (1967) found that component 8 could be released from whole casein by treatment with EDTA by heating and by the addition of ammonium sulfate, processes which disrupt casein micelles.

Furthermore, the fact that the proteose-peptones liberated from washed casein micelles contain phosphorus implicates their casein origin. A large portion of the components comprising this fraction seem to be located in the micelle, and are easily liberated upon acidification to pH 4.6. Possibly they occupy periphery positions in the micellar structure. This would explain the presence of proteose-peptone components in both the serum and micellar casein, as demonstrated in the respective electrophoretic patterns in this study. β-casein seems to be loosely associated in the micellar structure since it readily dissociates from the micelle at temperatures lower than 8.5 C (Garnier, 1966).

Conceivably milk protease could cleave the caseins further than is presently acknowledged, forming very small peptides, such as the extremely small peptides evidenced in this study.

Since proteose-peptone zones were present in the casein-free, centrifuged serum specimen and not in the electrophoretic patterns of the proteose-peptone specimen liberated from the casein micelle, not all of the proteose-peptone components arise from micellar casein. Ng *et al.* (1970) demonstrated that component 3 is solely a serum

constituent, which was verified in this study. Kang (1971), employing double diffusion immunological techniques, showed homology between some of the proteose-peptone components and those of bovine serum.

The heat stability of proteose-peptone components that are of serum origin could be accounted for by postulating that they are rendered heat-stable by virtue of glycosylation and/or phosphorylation.

Suggested Experiments to Further Examine the Proteose-Peptone Fraction

Increased electrophoretic resolution might be achieved by two-dimensional electrophoresis.

Casein could be treated with milk protease, or plasmin, and the products subjected to heating at 95 C for 30 min, and then acidified to pH 4.6. This would yield those breakdown products that are actually proteose-peptone like. Examination of these products by electrophoretic methods employed in this study would verify whether they are, indeed, analogous to the proteose-peptone components.

To assess whether proteose-peptone components are formed in the mammary gland by milk protease, or plasmin, action or after milking has occurred, milk could be stored for varying periods of time, and the changes, if any, in the proteose-peptone fraction monitored by gel electrophoresis.

Immunoelectrophoresis could be a further means of determining whether the proteose-peptone components arise from micellar casein, milk serum, bovine serum, and/or the plasmalemma.

The most important work to be done involves isolating the many components, in pure form, so that structural work can be pursued. Preparative gel electrophoresis and anionic exchangers could be used

as a means of isolation. Concanavalin A could be used, in an immobilized form, to separate the carbohydrate-containing proteins from those which are not glycosylated.

N- and C-terminal determinations and immunoelectrophoresis could be used to test the homogeneity of isolated proteins, and once a pure protein has been isolated, various structural methods could be employed. The determination of the amino acid sequence would be the ultimate structural method used, along with the determination of whether the carbohydrate linkages to the native protein are N-glycosidic or O-glycosidic, and the exact number and location of the linkages of the polypeptide chain.

SUMMARY

The implementation of increasing gel concentrations in PAGE in a discontinuous buffer system to fractionate the proteose-peptone fraction of bovine milk demonstrated that at least 62 different components comprise this fraction. This enhanced resolution is presumed to be due to the separation of this fractions' components more on the basis of size than on charge. The molecular weight species observed in this fraction ranged from <3000 to ~90,000 daltons, with a majority of components in the range of 31,000 daltons and below. As many as 23 zones were observed in IEF-PAG with apparent pI's ranging from 3.8 to 5.3. Phosphoproteins, glycoproteins, and phosphoglycoproteins were detected. Component 3 was found to be of serum origin, whereas components 5 and 8 were shown to be casein-associated and distributed between the casein micelle and the serum. All three classical components were heterogeneous.

The concept that proteose-peptone components arise from the proteolytic action of milk protease or plasmin on β-casein was reinforced.

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APPENDIX

APPENDIX

Table A-1. Buffer solutions used in this study.

PAGE in a Discontinuous Buffer System

.062M Tris-HCl, pH 6.7

Dissolve 1.877 g Tris in 200 ml distilled, deionized water. Add 1N HCl until pH 6.7 reached. Make to 250 ml.

.380M Tris-HCl, pH 8.9

Dissolve 46.018 g Tris in 900 ml distilled, deionized water. Add 1N HCl until pH 8.9 reached. Make to 1000 ml.

.046M Tris-glycine, pH 8.5

Dissolve 11.132 g Tris and 57.6 g glycine in 1800 ml distilled, deionized water and make to 2000 ml.

SDS-PAGE

.40M phosphate buffer, pH 7.2, containing 0.2% SDS

Dissolve 15.6 g $NaH_2PO_4.H_2O$ and 77.2 g $Na_2HPO_4.7H_2O$, and 2.0 g SDS in 900 ml distilled, deionized water and make to 1000 ml.

.20M phosphate buffer, pH 7.2, containing 0.2% SDS

Dissolve 15.6 g $NaH_2PO_4.H_2O$ and 77.2 g $Na_2HPO_4.7H_2O$, and 4.0 g SDS in 1800 ml distilled, deionized water and make to 2000 ml.

.01M phosphate buffer, pH 7.2, containing 3% SDS, 1% 2-mercaptoethanol

Dissolve 6.00 g SDS and 200 µl 2-mercaptoethanol in 10 ml .20M phosphate buffer, pH 7.2. Make to 200 ml with distilled, deionized water.

Table A-2. Formulation of the polyacrylamide gels used in this study.

PAGE in a Discontinuous Buffer System

5% spacer gel solution

Dissolve 1.1875 g acrylamide and .0625 g bisacrylamide in 20 ml .062M Tris-HCl buffer, pH 6.7, and make to 25 ml.

X% running gel solution

Dissolve .475X g acrylamide and .025X g bisacrylamide in 40 ml Tris-HCl buffer, pH 8.9, and make to 50 ml.

Polymerization of Gel

50 ml gel solution

50 µl TEMED

.20 ml 2.0% ammonium persulfate (freshly prepared)

SDS-PAGE

X% (5%-12.5%) gel formulation

10.00 ml .20M phosphate buffer, pH 7.2

.80X ml stock gel solution (23.750 g acrylamide and 1.250 g bisacrylamide dissolved in 100 ml).

1.00 ml 0.5% ammonium persulfate (freshly prepared)

20 µl TEMED

Y ml distilled, deionized water (to bring to 20 ml)

17.5% gel formulation

12.5 ml .40M phosphate buffer, pH 7.2

29.2 ml stock gel solution (28.500 g acrylamide and 1.500 g bisacrylamide dissolved in distilled, deionized water and brought to 100 ml)

50 µl TEMED

1.50 ml 0.5% ammonium persulfate (freshly prepared)

Y ml distilled, deionized water (to bring to 50 ml)

IEF-PAG

8.00 ml gel solution (1.240 g acrylamide and .0125 g bisacrylamide dissolved in distilled, deionized water and brought to 10 ml)

1.00 ml riboflavin (w/v)

.75 ml 0.8% TEMED (v/v)

125 µl ampholine pH 3.0-5.0

125 µl ampholine pH 4.0-6.0

PAGE-Gradient

X% in 2% sucrose--low concentration

Dissolve .95X g acrylamide, .05X g bisacrylamide, and 2.00 g sucrose in 80 ml .380 M Tris-HCl buffer, pH 8.9, and make to 100 ml.

Y% in 15% sucrose--high concentration

Dissolve .95Y g acrylamide, .05Y g bisacrylamide, and 15.00 g sucrose in 80 ml .380M Tris-HCl buffer, pH 8.9, and make to 100 ml.

Gradient Formulation

75 ml low concentration

75 µl TEMED

1.30 ml 1.0% ammonium persulfate (freshly prepared)

--into left-hand chamber of gradient-maker

70 ml high concentration

70 µl TEMED

.60 ml 1.0% ammonium persulfate (freshly prepared)

--into right-hand chamber of gradient-maker

Table A-3. Staining solutions used in this study.

Coomassie Brilliant Blue R--.25% (w/v)

Dissolve 1.25 g Coomassie Brilliant Blue R in 46 ml glacial acetic acid and 227 ml methanol. Make to 500 ml with distilled, deionized water.

Coomassie Brilliant Blue G--.04% (w/v) in 3.5% $HClO_4$

Dissolve .400 g Coomassie Brilliant Blue G in 35 g $HClO_4$. Make to 1000 ml with distilled, deionized water.

"Stains-all"--Stock Solution

Dissolve .100 g "Stains-all" in 75 ml formamide. Make to 100 ml volume.

"Stains-all"--Working Solution

Mix 10 ml of "Stains-all" stock solution with 10 ml formamide, 20 ml isopropanol, and 1.0 ml 3.0M Tris-HCl. Make to 200 ml with distilled, deionized water.

Table A-4. Staining procedures used in this study.

Coomassie Brilliant Blue R

Immerse gels in 5% TCA for at least 30 min. Stain in Coomassie Brilliant Blue R for at least two hours. Destain in (5:7:88 : methanol:acetic acid:water).

Coomassie Brilliant Blue G

Immerse gels in Coomassie Brilliant Blue G. Zones evidenced within 1-2 min. No destaining necessary. Enhance zone intensity at expense of background by immersion in 7% acetic acid.

Zacharius Method of Staining Glycoproteins

1. Immerse in 5% TCA for at least 30 min.
2. Rinse lightly with distilled, deionized water.
3. Immerse in H_5IO_6 (in 3% acetic acid) for 50 min.
4. Wash overnight with distilled, deionized water with several changes.
5. Immerse in fuchsin-sulfite stain for 50 min in dark at 4 C.
6. Wash with 0.5% freshly prepared $K_2S_2O_5$ 3 times for 10 min each.
7. Wash with distilled, deionized water until desired staining level is achieved.

Phosphoprotein Staining--"Stains-all" Procedure

Fix gels in 25% isopropanol for at least two hours. Stain gels in "Stains-all" overnight in dark, or until desired intensity achieved. Excessive staining causes dark background.

Table A-5. Chemicals used in this study and their sources.

Chemical	Source
Acrylamide	Ames, BioRad
Bisacrylamide	Ames
SDS (Sodium dodecyl sulfate)	BioRad
TEMED (Tetraethylmethylenediamide)	BioRad
Photoflo	Eastman
Stains-all	Eastman
Boric acid	Fisher
Bromophenol blue	Fisher
Glycine	Fisher
Potassium metabisulfite	Fisher
Schiff reagent	Fisher
Periodic acid	G. Frederick Smith
BDH standard proteins	Gallard-Schlesinger
Ammonium persulfate	J. T. Baker
Sucrose	J. T. Baker
Ampholine	LKB
Acetic acid	Mallinckrodt
Cupric sulfate	Mallinckrodt
Hydrogen peroxide	Mallinckrodt
Riboflavin	Mallinckrodt
Selenium dioxide	Mallinckrodt
Sodium hydroxide	Mallinckrodt
Sulfuric acid	Mallinckrodt
TCA (trichloroacetic acid)	Mallinckrodt

2-mercaptoethanol	Matheson, Coleman and Bell
Chymotrypsinogen	Nutritional Biochemical Corp.
Bovine serum albumin	Pierce
RNA polymerase--β, β'-subunits	Pierce
Coomassie Brilliant Blue R	Sigma
Coomassie Brilliant Blue G	Sigma
L-glutamic dehyrogenase	Sigma
Insulin	Sigma
β-lactoglobulin	Sigma
Ovalbumin	Sigma
Phenol red	Sigma
Ribonuclease	Sigma
Tris (trishydroxymethylaminomethane)	Sigma

ADDENDUM

ADDENDUM

Andrews (1978) concluded that proteose-peptone components 5 and 8-fast are, respectively, the 1-105 and 1-28 segments of β-casein A^2 formed by the cleavage of β-casein to form γ^2-casein and γ^1-casein.

Andrews heated skimmilk at 95 C for 30 min, adjusted the milk to pH 4.6, collected the supernatant, and saturated with $(NH_4)_2SO_4$. The precipitate was fractionated by dialysis to give the diffusible proteose-peptone component 8-fast and by gel filtration to give component 5 of the proteose-peptone fraction. The amino acid analyses of components 5 and 8-fast demonstrated a very close correlation to the 1-105 and 1-28 segments of β-casein A^2, respectively (see Addendum Table).

Kanno and Yamauchi (1978) postulated that proteose-peptone component 3 is identical to a soluble glycoprotein (SGP) of the milk fat globule membrane. They employed Ouchterlony's double immunodiffusion and Scheidegger's immunoelectrophoretic assays to assess any antigenic similarities between the SGP fraction and individual proteose-peptone components 3, 5, and 8. Only component 3 contained the anti-SGP reacting protein. Even though their SDS-PAGE patterns were somewhat different, the SGP and component 3 each contained a major

glycoprotein of 20,000 daltons, which seemed to cause the identical antigenicity of both protein fractions.

Andrews, A. T. 1978. Proteolysis in milk and the formation of proteose-peptones. XX: Int. Dairy Cong.

Kanno, C. and Yamauchi, K. 1978. Antigenic similarity between a major soluble glycoprotein fraction of milk fat globule membrane and proteose-peptone fraction of milk. XX: Int. Dairy Cong.

Table Addendum.--Amino acid analyses (mol/mol protein).

Amino acid	Component 5	β-casein A[2] (1-105)	Component 8-fast	β-casein A[2] (1-28)
Aspartic acid	6.0	6	2.1	2
Threonine	4.7	5	1.3	1
Serine	7.3	9	4.2	5
Glutamic acid	24.2	24	6.8	7
Proline	13.0	14	1.7	1
Glycine	3.0	3	1.0	1
Alanine	2.7	3	0.2	0
Valine	7.7	9	2.0	2
Methionine	2.3	2	0.3	0
Isoleucine	6.1	7	2.6	3
Leucine	8.3	8	2.9	3
Tyrosine	1.8	1	0.3	0
Phenylalanine	4.2	4	0.2	0
Histidine	1.8	1	0.2	0
Lysine	6.9	7	0.9	1
Arginine	1.9	2	1.9	2

[a]From Andrews (1978).