FRACTIONATION AND CHARACTERIZATION OF SOLUBLE AND INSOLUBLE PROTEINS OF THE MILK FAT GLOBULE MEMBRANE

By

Carl Thomas Herald

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Approved J. Robert Brunner

ABSTRACT

CARL T. HERALD

Isolation procedures and some physical and chemical characteristics of the fat-globule membrane proteins were studied. Five concentrations of cold ethanol, four of cold n-butanol, and room temperature n-butanol were investigated as agents to disrupt the membrane lipoprotein complex. Membrane proteins pretreated with 35 percent ethanol (final concentration about 26 percent) were most amenable to physico-chemical studies. Cold ethanol treatment was more satisfactory than cold or room temperature n-butanol as a pretreatment for disrupting the membrane lipoprotein complex.

Based upon solubility in 0.02 M sodium chloride solution, the membrane proteins were separated into soluble and insoluble fractions. The soluble fraction had a strong Molisch reaction, reduced Fehling's solution subsequent to mild acid hydrolysis, was sulfhydryl negative after heating to 75°C., and had nitrogen values varying with preparation from 9.5 to 11.5 percent. An average of seven-fold more phosphatase activity was found in the soluble than in the insoluble fraction. A single skewed component was observed in alkaline buffers when the soluble fraction was studied electrophoretically. For these components, regression equations $Y = 8.840-1.762X$, $Y = 7.250-1.455X$, and $Y = 4.663-0.900X$ were found which gave isoelectric points at pH 5.02, 4.98, and 5.18 respectively.

ABSTRACT CARL T. HERALD

On the basis of sedimentation velocity data, insolubility in half-saturated ammonium sulfate, and an isoelectric zone near pH 5.0, the soluble proteins were tentatively classified as globulin in nature.

The residual fraction was insoluble in dilute acids, bases, 25 percent sulfuric acid, and 6 or 8 molar urea solutions. Sodium sulfide and sodium lauryl sulfate were representative solubilizing agents. Nitrogen values ranged with preparations from 12.9 to 13.9 percent. A qualitative sulfhydryl-positive reaction was obtained upon heating the protein moiety to 75° C. The insoluble fraction contained 5.6 times more iron, 25 times more molybdenum, and 10 times more xanthine oxidase activity than the soluble fraction. Electrophoretically, sodium sulfide-solubilized insoluble protein showed one homogeneous component and detergent-solubilized protein had two prominent and one minor components.

On the basis of insolubility in the usual protein solvents, reactivity to specific reducing agents, and amino acid composition, the insoluble proteins were provisionally classified as pseudokeratin.

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INTRODUCTION

Milk fat exists in milk in the form of tiny globules surrounded by a complex of proteins and lipid materials. These globules, which average three to four microns in diameter, are coarsely dispersed and in time, will rise to the top of the serum, due to a lower specific gravity, to form a cream layer. The function of the membrane surrounding the fat globules is of considerable fundamental and practical interest.

In particular, the protein portion of the membrane has been a source of wide disagreement among researchers. Certain scholars of dairying chose to believe that no membrane existed, even after a membrane-like material had been demonstrated. Several attempts have been made to identify the membrane proteins with casein or whey proteins. Some have suggested that a unique protein surrounds the globules which defies existing classification. More recently, a hypothesis was presented which suggested that fat globules are surrounded by a continuous protein layer to which are adsorbed microsomes.

There are data in the literature which totally or partially refute the above notions concerning the membrane proteins. For instance, an electrophoretic study showed that the so-called membrane protein is heterogeneous.

The origin of the membrane is still in the stage of speculation. There are no data available showing the time at which fat globules are covered with the membrane material.

Although these considerations are in the realm of the physiologist, it would seem that a study of the membrane proteins with the currently available chemical and physical techniques should yield data suggesting a logical classification and source of these proteins.

In practice, the fat-globule membrane has been shown to be intimately associated with copper-induced oxidized flavors, rancidity in milk, and with problems dealing with de-emulsification of the fat globules. Thus, it is clear that chemical and physical data related to the milk fat globule should be significant from a fundamental and practical standpoint. Some of the problems and considerations inherent to the membrane proteins are presented in this manuscript.

The development of the adapted procedure is reported under the exploratory investigation section. In the procedure section are given the methods used to prepare and characterize the soluble and insoluble fractions from the membrane proteins.

REVIEW OF LITERATURE

Historical

A considerable amount of study has been devoted to the chemistry and physical phenomena associated with the "membrane" of the milk fat globule. Recently, King (1955) has written an excellent, comprehensive review in this area of research.

Ascherson (1840) was the first to postulate that a membrane surrounded the milk fat globules. In his experiments, olive oil in contact with egg white, acquired a membrane which Ascherson called "haptogen." He reasoned that milk fat should behave in a similar manner in milk. Babcock (1869) believed that a similarity existed between the coagulation of blood and the creaming of milk and reported that fat globules were surrounded by fibrin. This hypothesis was later disproved by Hekma (1922). Storch (1897) introduced the technique of washing cream to remove materials not closely associated with the fat globules and postulated that a mucin-like protein surrounded the fat globules. Abderhalden and Völtz (1909) reported that the protein portion of the membrane was not casein. Palmer and Samuelson (1924) indicated that the emulsion-stabilizing substances adhering to the fat globules in cow's milk consisted of a single globulin-like protein and a mixture of phosphatides of undetermined nature.

Employing a rather unique isolation procedure, Hattori (1925) isolated a proteinaceous membrane material which he

called "haptein." Haptein was unlike any other known milk protein in respect to solubility, sulfur, and cystine content. He suggested that haptein was a keratin-like protein. Titus, Sommer and Hart (1928) concluded that the membrane protein was quite similar to casein. These workers suggested that their preparation was contaminated with some unknown substance since the isolated protein would not dissolve in 0.5 N sodium hydroxide. Rahn and Sharp (1928) believed that the foaming property of milk was due to "schaumstoff," which was thought to be a protein closely associated with the fat globules. Furthermore, schaumstoff was reported to be completely insoluble. Weise and Palmer (1932) prepared a series of artificial emulsions of butterfat stabilized with either calcium caseinate, whey proteins, or lecithin prepared from egg yolks and concluded that none of the substances constituted the sole material adsorbed on the surface of the fat globules in normal cow's milk.

Palmer and Weise (1933) showed that the protein of the membrane was closely associated with phospholipides and high-melting triglycerides. Hot ethyl alcohol and ethyl ether were used to extract the lipid materials from the membrane protein. Attempts to place the protein in solution resulted in cloudy, milky suspensions. Furthermore, the physical properties, as well as the nitrogen, sulfer, and phosphorus content of this preparation, did not correspond with similar characteristics of known milk proteins. These investigators obtained 0.66 to 0.89 grams of membrane material per 100 grams of milk fat. In a later publication, Weise and Palmer

(1934) reported the Van Slyke nitrogen distribution for the membrane proteins. Rimpila and Palmer (1935) found that the membrane contained phosphatase which was not completely removed after the cream had been washed several times. Palmer and Tarassuk (1936, 1940) and Tarassuk and Palmer (1939) studied a series of artificial milks in an attempt to explain certain surface phenomena associated with the milk-fat membrane.

According to Pedersen (1936), separator slime contained an insoluble material which he assumed to be related to casein. Jack and Dahle (1937a, 1937b) and Dahle and Jack (1937) employed an electrophoretic technique to study the fat globules of milk. Electrokinetic mobilities for fat globules in acetate and citrate buffers revealed an isoelectric point at pH 4.3. Furthermore, the data suggested the probability of a double layer of phospholipide and protein on the surface of the fat globules. Sandelin (1941) isolated an ammonia-insoluble protein material from milk which he thought to be identical with the membrane protein. The insoluble material was isolated by mixing milk or cream with 25 percent ammonia and alcohol which was followed by centrifugation. Sandelin reported a sulfur content of 0.57 percent which is much lower than the values reported by Hattori (1925), Weise and Palmer (1934), and Hare, Schwartz and Weese (1952).

Palmer (1944), in his review, postulated that the membrane consisted of a special group of substances whose origin might be due to their greater capillary activity. Jenness and Palmer (1945a) demonstrated that the ratio of protein to

phospholipide was not identical in buttermilk and butter serum. The butter serum fraction contained a decidedly greater amount of phospholipide per unit of protein.

Mulder (1949) believed that the surface layer must not be regarded as having been adsorbed from the milk plasma, but that the membrane consisted of components of the protoplasm of the cells of the lactiferous glands and of substances adsorbed from the milk plasma. Hare et al. (1952) studied the amino acid composition of the membrane materials and found more arginine, glycine and phenylalanine and less aspartic acid and leucine were in the membrane protein than in other milk proteins. Brunner, Duncan and Trout (1953a) used cold alcohol with subsequent ether extractions to purify the membrane proteins. On the basis of nitrogen content, these treatments were as effective in removing lipid as that of earlier investigators who used hot organic solvents.

An electrophoretic study of the membrane proteins by Brunner, Lillevik, Trout and Duncan (1953c) revealed as many as four components. The electrophoretic patterns indicated that the protein components were of a closely associated nature. On the basis of further study with an ultracentrifuge, Brunner, Duncan, Trout and MacKenzie (1953b) reported one principal component, $S_{20} = 7.3$, which suggested that the membrane protein was "globulin" in nature.

According to Morton (1954), milk fat globules are surrounded by a continuous protein membrane on which are adsorbed microsomes. Electron microphotographs were presented to support his theory. Based on electrophoretic studies,

Sasaki, Koyama and Nishikawa (1956) concluded that the fat membrane material was similar to whey proteins less beta-lactoglobulin. Sasaki and Koyama (1956) postulated that the fat membrane does not contain casein or beta-lactoglobulin but a part of the whey proteins plus a possible unknown or specific protein.

Considerable attention has been devoted to certain substances associated with the membrane proteins. Certain proteins in equilibrium between the membrane-plasma interface as well as specific compounds of structural significance have been studied. Sharp (1940) estimated that the fat globules in a quart of Guernsey milk had a total surface area of about 1050 square feet. Trout, Halloran and Gould (1935) showed that homogenization increased the surface area of fat globules about five or six times. Trout (1950) presented additional information concerning the role of surface area in normal and homogenized milk.

The fact that cream which was separated from cold milk contained an agglutinating material not commonly found in the cream separated from warm milk was observed by Sharp and Krukovsky (1939). This material, capable of causing fat clustering, was isolated and identified as euglobulin by Dunkley and Sommer (1944). Peters and Trout (1945) reported that an attraction, based in part upon oppositely charged particles, existed between the fat globules and the leucocytes. The positively charged leucocytes were attracted the most at pH values near the isoelectric point of milk casein and the least at lower pH values. Sommer (1951) postulated that fat

globules exist in milk plasma surrounded by an atmosphere of reversibly adsorbed materials, "principally ions." The enzyme phosphatase, which is associated with the membrane, has been studied by Kay and Graham (1933), Morton (1954), and Zittle, DellaMonica, Custer and Rudd (1956b). Morton (1950) and Zittle and DellaMonica (1952) reported that n-butanol had the specific ability to induce the separation of lipoprotein complexes. Morton (1950) treated buttermilk from washed cream with n-butanol and obtained a 5000-fold increase in the concentration of phosphatase, as compared with milk. Other materials reported to be associated with the membrane include xanthine oxidase, diaphorase, DPN-cytochrome c reductase and approximately 11 percent nucleic acid (Morton, 1954). Zittle et al. (1956b) employed similar analytical methods and found less than 0.5 percent nucleic acid associated with the membrane. Further, they theorized that microsomes contained protein, phospholipide, and nucleic acid which might be pictured as a parcel of enzymes cemented together with phospholipides and nucleic acid.

Toyama (1932), Ball (1939), Polonovski, Baudu and Neuzil (1949), Morton (1954), Avis, Bergel and Bray (1955) and Zittle et al. (1956b) have shown that the fat globule membrane is rich in xanthine oxidase. The association of iron and copper with the fat-globule membrane was shown by Davies (1933). Kon, Mawson and Thompson (1944) stated that carotenoids and cholesterol were present in large amounts when the ratio of the fat-globule surface to fat was high. White, Eaton and Patton (1954) assumed that carotenoids were in a

dilute solution or in a loose chemical complex on the globule surface. The carotenoid concentration in the phospholipide membrane was calculated to be 0.0645 percent as compared to 0.000476 percent in the interior of the fat globule.

Palmer and Weise (1933) demonstrated that phospholipides were associated with the fat membrane. Kurtz, Jamieson and Holm (1934) isolated an ether-soluble lecithin-cephalin fraction (about 56 percent lecithin and 44 percent cephalin) which contained 70.6 percent oleic acid. The role of phospholipides in dairy products was demonstrated by Thurston, Brown and Dustman (1936). These investigators showed that phospholipides were strong contributors to the so-called "rich flavor" of milk. Moreover, the oxidized flavor called "oily-stale" was shown to be intimately associated with phospholipides. A technique for the preparation of phospholipides developed by Olson (1944) yielded one gram of phospholipide from about 1500 pounds of milk. Jenness and Palmer (1945b) reported a high-melting triglyceride in close association with the fat membrane.

Dahle and Pyenson (1938), Gould and Sommer (1939), and Townley and Gould (1943) have demonstrated the presence of sulfhydryl groups from heated membrane proteins. According to Townley and Gould (1943), heat labile sulfides of milk originate from two sources: first, the milk serum, and second, the material associated with and firmly attached to the fat globules. The effect of heating cream prior to the washing procedure upon the subsequent recovery of membrane protein was demonstrated by Loewenstein and Gould (1954).

Milk which was heated to 40°C. momentarily, 62°C. for 30 minutes, and 82°C. for 15 minutes yielded membrane material that contained 21.86, 15.54, and 7.70 percent protein, respectively.

Commentary and Objectives

The literature yields little specific information which would permit a logical classification of the membrane proteins, but theories concerning the nature and origin of the membrane constituents are abundant. Unfortunately, some of these theories are based on a few or no laboratory observations. The complexity of the membrane has undoubtedly encouraged some workers to seek areas of more lucrative data.

Recently published reports propose that the protein portion of the fat membrane is of cellular origin, which excludes casein and the whey proteins. The application of electrophoresis and ultracentrifugal techniques to the study represent the best approach available at this time. A vexing problem confronting investigators interested in this problem has been the insolubility of a fraction of the membrane. Obviously, the techniques mentioned previously are of limited value unless the materials in solution are representative samples. The primary objective of this investigation was to devise a technique which would render the membrane proteins soluble. A minimum of change was desired for proteins to be used in physico-chemical studies. Subsequent to this accomplishment it was hoped to characterize the membrane proteins by several techniques. Thus, with more data available,

certain clues might be obtained which would reveal a better insight as to the origin, role and nature of the fat-globule membrane proteins.

EXPERIMENTAL PROCEDURE

The milk used throughout this study was from a mixed herd source. Fresh, raw milk of about 3.5 percent fat and 12.3 percent total solids was obtained from the Michigan State University Creamery.

Exploratory Investigations

There is paucity of information in the literature on methods to render the membrane proteins soluble. Thus, a series of experiments were planned in which existing methods were reviewed and new ideas investigated. Washed-cream buttermilk (hereafter referred to as serum) was obtained from washed cream prepared according to classical methods (Storch, 1897; Weise and Palmer, 1933; and Brunner et al., 1953a).

Concentration of the membrane material. Four methods based on different physical and chemical principles were investigated: 1) The condensing procedure of Brunner et al. (1953a), 2) Isoelectric precipitation at pH 3.9, according to Palmer and Weise (1933), 3) Freezing the serum slowly to concentrate the membrane materials, and 4) Salting-out the proteins from the serum with 50 percent ammonium sulfate.

Removal of lipid from the native membrane. Concentrated membrane materials were treated with varied concentrations of cold (0° to -5°C.) ethanol or n-butanol. Alcohols were removed by washing the cold mixture several times with cold

ethyl ether on No. 192 Eaton and Dikeman folded filter paper. Several extractions with 30°C. ether were required to remove most of the lipid materials. The residual proteins were dialyzed, lyophilized, and stored in the dry state at -20°C. The intermittent freezing and thawing method of McFarlane (1942) was also investigated.

Solubility studies. One-tenth gram of dry protein was added to phosphate buffer of pH 7.6. The buffer was composed of 0.02 M phosphate and 0.15 M sodium chloride. After standing for 24 hours at 5°C., these solutions were centrifuged for 40 minutes at 12,800 G in a Model SS-1 Servall Centrifuge. Protein solubility was determined as soluble protein nitrogen according to the modified Kjeldahl method of Menefree and Overman (1940). The results were expressed as milligrams percent of nitrogen.

Lipid content of the lyophilized residual proteins. A modified procedure of Mojonnier and Troy (1925) was used for this purpose. Fifty milligrams of membrane protein were weighed into a 15 milliliter centrifuge tube and treated with 1 milliliter hot water, a few drops ammonium hydroxide, 0.2 milliliter ethanol, and 5 milliliters ethyl ether. The mixture was agitated and the supernatant was removed after centrifuging for approximately two minutes. The residue was extracted twice with ethyl ether before drying and reweighing. Loss in weight was considered to be lipid material.

Extraction of the dry residual membrane proteins with either veronal buffer solution or water. Lipid-extracted,

lyophilized membrane proteins were dispersed in either veronal buffer solution or tap water. The soluble materials taken up in the aqueous phase were separated from the mixture by centrifugation at 25,000 G for 30 minutes. The supernatant was lyophilized and dissolved in selected buffer systems for standard electrophoretic analysis.

Procedure for the Isolation of Fat-Membrane Proteins

The methods selected for the isolation and characterization of the membrane proteins were based on the experiences of other researchers and our own modifications. Simplicity, the element of time, and possible undesirable changes in the protein system were prime considerations in the development of the isolation procedure.

Preparation of the fat-globule membrane proteins. The fat-globule membrane proteins were prepared according to the flow diagram shown in Figure I. The cream-washing procedure was originated by Storch (1897) and refined by Weise and Palmer (1932). Brunner *et al.* (1953a) introduced the concept of cold ethanol treatment as a pretreatment for lipid removal. Concentration of the membrane material from serum by salting-out with ammonium sulfate and subsequent fractionation based on solubility in 0.02 *M* sodium chloride were adapted from exploratory studies.

Thirty percent cream was prepared from fresh, raw milk separated at 46°C. with a Model 518 DeLaval Laboratory Separator. The cream was washed six times with three volumes of 40°C. tap water which produced a washed cream of about 55

percent fat. After standing overnight at 5°C., the washed cream was churned in a glass jar agitated horizontally with a mechanical device. Butterfat was removed by warming the churned mixture to about 40°C. and separating with a DeLaval laboratory separator. The fat-membrane material was salted out of the serum at room temperature by adding slowly with agitation a saturated solution of ammonium sulfate until a final concentration of 55 percent ammonium sulfate was reached. Concentration of the membrane material was achieved by centrifuging at 25,000 G for 30 minutes in a Model SS-1 Servall Centrifuge. Fifty grams of protein-containing material were treated with 100 milliliters of 35 percent ethanol in ether which resulted in a final ethanol concentration of about 26 percent. The temperature was held at 0° to -5°C. while the mixture was agitated for ten minutes. Alcohol was removed by filtration in a refrigerated room (-20°C.) followed by five washings with 200 milliliters of cold ether. Finally the material was extracted three times with 250 milliliters of ethyl ether at 30°C. The proteins were made into a thick slurry with 0.02 M sodium chloride solution and held overnight under 29 inches of vacuum to remove residual ether. The resulting protein concentrate was separated into soluble and insoluble fraction by extracting with four 150 milliliters of 0.02 M sodium chloride solution and subsequent centrifugation at 25,000 G for 30 minutes. The supernatant contained the "soluble" fraction, whereas the pellet in the bottom of the centriguge tube was designated as the "insoluble" fraction. The protein solutions were dialyzed further

against 0.02 M sodium chloride solution and stored at 5°C.

Analytical Methods

Nitrogen. The method of Menefree and Overman (1940) was modified for this purpose. A fifty milligram sample of protein was digested slowly for about two hours with ten milliliters (N-free) sulfuric acid, 0.14 grams of mercuric oxide, and two grams of sodium sulfate. Following digestion 70 milliliters of distilled water and 30 millliliters of 50 percent sodium hydroxide containing sodium thiosulfate were added. The mixture was steam distilled for ten minutes into 25 millliliters of boric acid solution containing four drops of indicator. This was titrated with approximately 0.05 N sulfuric acid from a burette calibrated to 0.05 millliliters.

Phosphorus. The protein samples were digested by modifying the procedure of Horecker, Ma and Haas (1940). The method of Fiske and Subbarow (1925) was used for color development. Fifty milligrams of the protein preparation were digested to a brown color with five millliliters of 10 N sulfuric acid after which five drops of 30 percent hydrogen peroxide were added and the solution heated to clearness. A standard curve was prepared by plotting optical density against known phosphorus concentrations. After appropriate dilutions and color development, the phosphorus content of the unknown samples was read directly from a prepared standard curve.

Sulfhydryl groups. The method of Josephson and Doan (1939) as modified by Patton and Josephson (1949) was used for this purpose.

Fat and total solids. Total solids and fat were determined on milk, cream, and washed-cream buttermilk by the method of Mojonnier and Troy (1925).

Amino acids. The unidimensional, buffered paper chromatographic technique of McFarren (1951) and McFarren and Mills (1952) was adapted for this purpose. The conditions for the amino acid procedure are summarized in Table 1. The chromatograms were irrigated in a Chromatocab, Model A300 (Research Equipment Corporation) on Whatman No. 1 filter paper. The standard solutions contained 0.4 microgram amino acid per microliter, except for tyrosine, which contained 0.3 microgram per microliter. The known and unknown solutions were delivered on the chromatographic sheet in 5 microliter portions. Four concentrations of the standard solution (5, 10, 15, and 20 microliters) were applied. A standard curve was prepared for each amino acid and chromatogram. The borate buffer was modified to exclude potassium chloride and the phosphate buffer was modified to 0.02 M for the arginine-lysine run. After the color developed, the spots were cut out and eluted with 5 millliliters of a 1:1 mixture of isopropyl alcohol and water. Optical density was determined at 570 millimicrons in a Beckman Model B Spectrophotometer. Standard curves were prepared by plotting the concentration of the amino acid against the optical density. Amino acid concentrations in the unknown samples were read directly from the appropriate standard curve.

Ultraviolet analyses. A Beckman Model DK 2 Spectrophotometer was used to scan the protein solutions over the spectrum

range of 220 to 340 millimicrons. Protein concentration was 0.02 percent in 0.02 M sodium chloride solution.

Infrared analyses. Nujol mulls were scanned between salt plates from 2 to 15 microns with a Perkin-Elmer Model 21 Spectrophotometer.

Ash. Two-gram samples were placed in a muffle furnace at 550° C. for 12 hours.

Solubilization of the insoluble proteins. The protein materials which were not soluble in ordinary protein solvents were treated with the following types of reagents: dilute acids and alkali, strong acids and alkali, urea, sodium sulfide and thioglycolic acid, sodium lauryl sulfate, and dodecyl benzene sodium sulfonate. The insoluble proteins were suspended in 0.02 M sodium chloride solution in these experiments.

Electrophoresis. Electrophoretic mobility patterns were recorded at approximately 1°C. with a Perkin-Elmer Model 38-A Electrophoresis Apparatus originally described by Moore and White (1948) and subsequently equipped with a Philpot-Svennson cylindrical lens system as described by Longsworth (1946) and Bull (1951). Buffers were calculated with the Henderson-Hasselbach and ionic strength equations. In all cases, an ionic strength of 0.1 was used. The pK values were taken from Kolthoff and Laitinen (1948) and Longsworth (1952). The pH measurements on the buffers were taken with a Beckman Model G Potentiometer at 10, 15, 20, and 25°C. Temperatures were plotted as abscissas and pH values as ordinates, and pH values for 1°C. were obtained by extrapolation. One-half percent protein concentration was obtained by diluting the

original extracts with 0.02 M sodium chloride solution. Protein solutions were dialyzed for at least 24 hours at 5°C. against three 350 milliliter buffer changes. A mechanical stirrer (American Instrument Company) was used to facilitate dialysis. The photographic negatives of the electrophoretic images were placed in a photographic enlarger and the twice magnified image was traced on good quality graph paper. The distance from the initial boundary to the central area of each peak was measured for mobility calculations. Average values of the ascending and descending patterns were taken. To obtain precise mobilities, the conductivity at each boundary should be known. When this is impossible, best results are obtained by averaging ascending and descending values (Moore, 1949).

Potential gradient, commonly referred to as field strength, was calculated as follows: $F = i/aK$

where:

F = potential gradient,

i = current in amperes,

a = cross-sectional area of the U cell, and

K = the specific conductivity of the buffer protein solution.

Electrophoretic mobility (u) was calculated with the following formula: $u\ (cm.^2, volt^{-1}, sec.^{-1}) = \frac{dak}{tirm}$

where:

d = distance boundary traveled,

a = cross-sectional area of U cell,

k = conductivity cell constant,

t = time in seconds,

i = current in amperes,

r = resistance of buffer in ohms, and

m = magnification factor of optical system.

Compositional distribution was determined by dropping ordinates from the lowest point between peaks and subsequently weighing the respective cut-out areas. Average values of the ascending and descending pattern area were recorded.

TABLE 1

SUMMARY OF THE QUANTITATIVE, UNIDIMENSIONAL, BUFFERED PAPER CHROMATOGRAPHY PROCEDURE

Solvent	pH	Molarity	Amino acids determined	Running time	Drying time	Concentration of acetic acid in ninhydrin
				(hr.)	(min.)	(%)
Phenol	12.0	0.067	Aspartic acid Glutamic acid Serine Glycine Threonine Alanine	24	30	4
m-Cresol	8.4	0.067	Tyrosine Histidine Valine Methionine	40	60	2
2,4 Lutidine	6.2	0.020	Lysine Arginine	40	30	2
o-Cresol	6.2	0.067	Phenylalanine	24	60	2
Benzyl alcohol + n-butanol 1:1 ratio	8.4	0.067	Proline Isoleucine Leucine	24	30	4% in 0.4% isatin 2

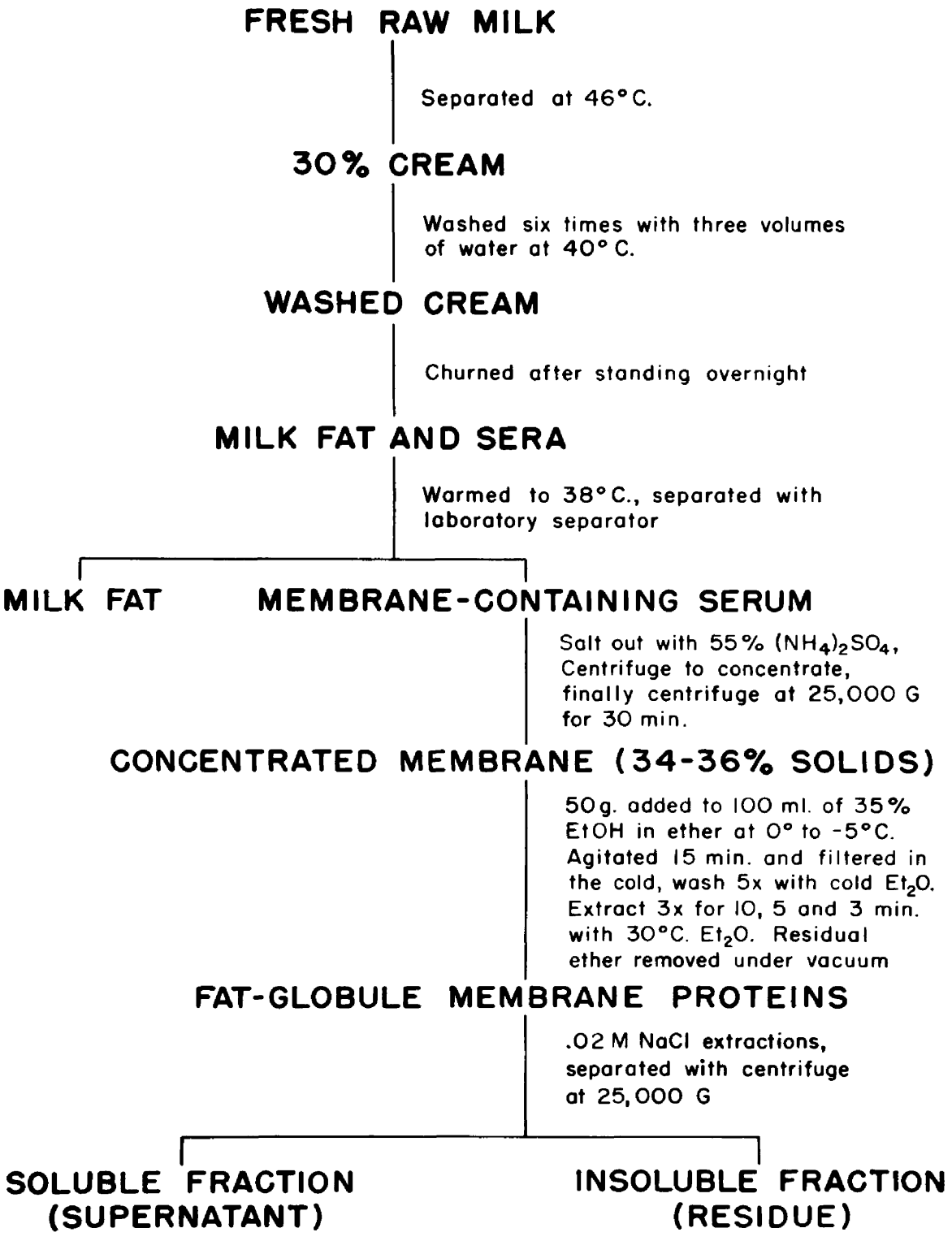


FIGURE I. Procedure for separating the fat-globule membrane proteins.

RESULTS

Exploratory Investigations

Effect of various alcohol pretreatments. The data in Tables 2 and 3 were obtained for the ammonium sulfate-precipitated material. These data show that membrane proteins pretreated with 35 percent ethanol had the highest nitrogen content and appeared to be the most soluble. Membrane proteins pretreated with cold n-butanol had lower nitrogen values, higher lipid content, and were less soluble than proteins treated with ethanol. Cold n-butanol tended to form unmanageable emulsions with the membrane material. The influence of cold ethanol treatments and room temperature n-butanol on the nitrogen content of the resulting membrane protein materials are shown in Table 3. Proteins treated with cold 35 percent ethanol contained 12.32 percent nitrogen, whereas proteins treated with room temperature n-butanol contained 12.17 percent nitrogen.

The brown color of the membrane proteins disappeared whenever the concentration of ethanol was greater than 35 percent. Ether which contained peroxides also bleached the protein materials. In both cases, xanthine oxidase activity was lost.

Electrophoretic characteristics. The protein materials which were used for this purpose were concentrated with ammonium sulfate and had the lipids removed with alcohol-ether treatments.

Previous to centrifuging, buffer solutions containing solvent-treated whole membrane proteins were milky and turbid. These solutions were resolved into optical clarity by centrifugation at 25,000 G for about 30 minutes. Invariably a brown residue was found in the bottom of the centrifuge tube.

Data in Figure II illustrate the effect of various ethanol pretreatments on the concentrated membrane proteins. These patterns showed qualitative similarities and indicated a closely associated two-component system. Electrophoretic mobility and relative component compositions are given in Table 8. A decrease in mobility with increased ethanol concentration was observed. The effect of room temperature n-butanol is shown in Figure II, row 6. On a qualitative basis, the n-butanol-extracted proteins appeared to be similar to the ethanol-extracted material, but the butanol-extracted material seemed to diffuse at a faster rate during the electrophoresis run.

Electrophoretic diagrams in Figure II, row 1, represent tap water soluble proteins which had been treated with 30 percent alcohol, extracted with ethyl ether, and lyophilized. The data indicate that tap water extracted the same protein materials as veronal buffer. The Miller and Golder (1950) buffer system in which sodium chloride supplies 90 percent of the ionic strength was used for the trial shown in Figure II, row 4.

TABLE 2

NITROGEN VALUES, LIPID CONTENT, AND SOLUBILITY DATA OBTAINED FOR MEMBRANE PROTEINS PRETREATED WITH VARIOUS CONCENTRATIONS OF EITHER COLD ETHANOL OR n-BUTANOL

Alcohol Treatment	Nitrogen	Lipid	Solubility
	(%)	(%)	(mg.% N)
Ethanol (25%)	11.13	3.7	54
Ethanol (35%)	12.54	4.0	56
Ethanol (55%)	11.56	7.8	35
Ethanol (95%)	12.11	5.6	48
n-Butanol (25%)	10.56	9.3	40
n-Butanol (35%)	9.79	17.3	38
n-Butanol (50%)	8.87	13.2	39
n-Butanol (100%)	10.47	10.9	42

TABLE 3

NITROGEN CONTENT OF MEMBRANE PROTEINS PRETREATED WITH COLD ETHANOL OR ROOM TEMPERATURE n-BUTANOL

Alcohol Treatment	Nitrogen
	(%)
Ethanol (30%)	11.42
Ethanol (35%)	12.32
Ethanol (95%)	12.12
Butanol (100%)	12.17

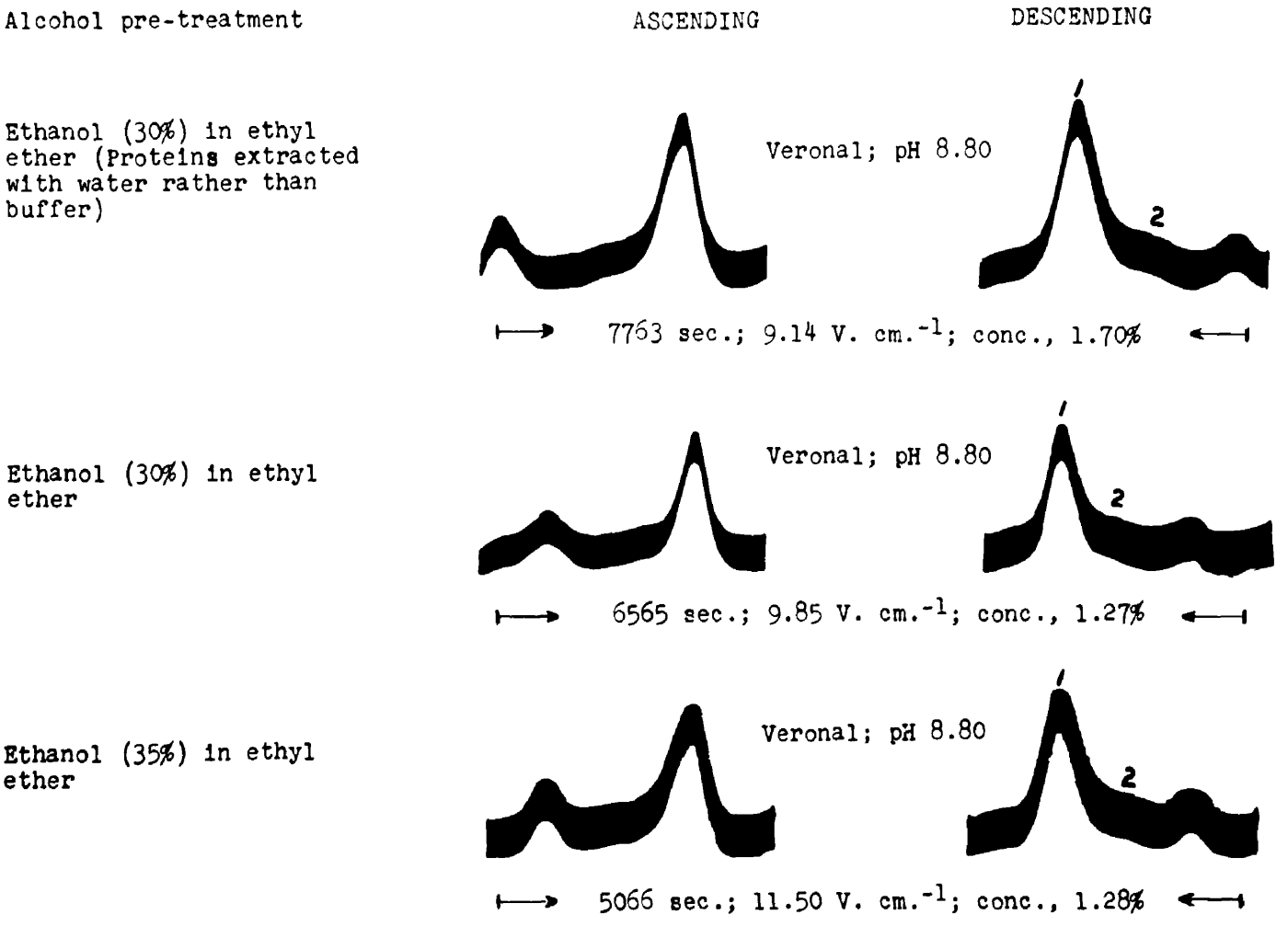


FIGURE II. The effect of alcohol pre-treatments and solvents on the electrophoretic mobility characteristics of the soluble membrane proteins.

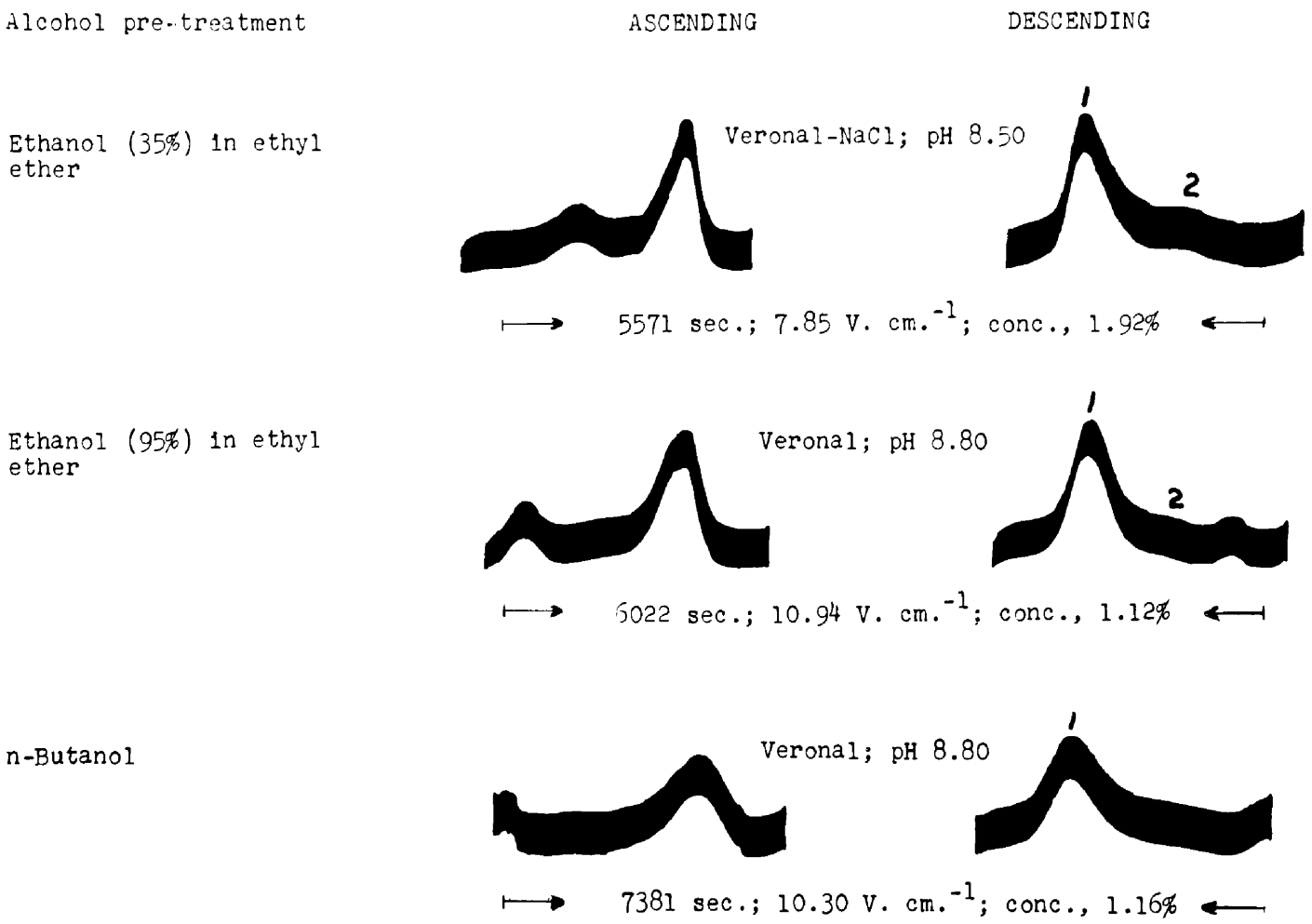


FIGURE II (Continued) The effect of alcohol pre-treatments and solvents on the electrophoretic mobility characteristics of the soluble membrane proteins.

Results of Physical and Chemical Studies

Exploratory results indicated that the proteins of the fat-globule membrane could be placed into solubility categories. The portion soluble in the supernatant of 0.02 M sodium chloride solution after centrifugation was called the soluble fraction, whereas the residue was designated as the insoluble fraction. The ratio of the soluble fraction to the insoluble fraction was approximately 44 percent and 56 percent, respectively. The data in the following section were used to characterize the soluble and insoluble fractions.

Solubility. The data in Table 4 illustrate the solubility characteristics of the membrane proteins. The soluble fraction was readily soluble in aqueous solvents but was readily salted-out by half saturation with ammonium sulfate. The insoluble material resisted solution in water, organic solvents, and alkaline buffers. Twenty-five percent sodium hydroxide, sodium sulfide in a sulfide/protein ratio of 0.6 : 1, sodium lauryl sulfate in a detergent/protein ratio of 0.8 : 1, and dodecyl benzene sodium sulfonate in a detergent/protein ratio of 1.2 : 1 were capable of effecting solution. Five milliliters of a 0.5 percent suspension of insoluble protein required 0.08 milliliters Tergitol 7 or 1.1 milliliters of Triton X-100 and momentary heat to 75°C. for solution. Solutions prepared with Tergitol 7 became turbid when cooled. Conversely, protein solutions which contained Triton X-100 remained turbid until cooled to 62°C. and heating the mixture induced turbidity. Protein solutions

prepared with Triton X-100 were quite viscous.

Enzymes. The data in Table 5 show the distribution of xanthine oxidase and alkaline phosphatase in the soluble and insoluble protein fraction of the membrane protein. Two samples prepared about one month apart were submitted for analyses. Enzyme activity was employed to indicate any undesirable changes that might occur in the protein system due to preparation technique. Xanthine oxidase was found to be concentrated in the insoluble fraction and phosphatase in the soluble portion.

Chemical composition. The data in Table 6 emphasize further the differences in chemical composition between the two fractions. The soluble fraction contained significantly more ash, phosphorus, magnesium, and calcium. The insoluble fraction contained a greater quantity of nitrogen and was sulfhydryl-positive after flash heating to 75°C.

Ultraviolet analyses. Absorption peaks in both fractions were most intense at 278 millimicrons. This is illustrated in Figures VII and VIII.

Infrared analyses. A spectrum representative of the soluble and insoluble fractions is shown in Figure IX. Absorptions attributable to the functional groups in the proteins were observed at 3.05, 6.05, and 9.50 microns. The spectrum of the soluble and insoluble fractions were almost fingerprint images, but the soluble portion revealed a small shoulder at 5.75 microns which was not evident in the insoluble portion.

Amino acids. The amino acid composition of the soluble and insoluble fraction is reported in Table 7. More valine, arginine, and methionine were found in the insoluble fraction than in the soluble fraction. Photographic reproductions of the developed chromatograms are presented in Figures XI to XV.

Electrophoresis. Electrophoretic patterns for the soluble fraction in acid and alkaline buffers are presented in Figure III. Various degrees of resolution were observed on either side of the isoelectric zone. On the acid side, two and three components were observed, but on the alkaline side, the components appeared more closely associated.

In Figure X, electrophoretic mobility was plotted as the ordinate and pH as the abscissa. The intercept of the regression curve with zero mobility established the isoelectric zone for the major components at about pH 5.0.

Figures IV and V represent electrophoretic mobility patterns of the insoluble protein material treated with various solubilizing agents. Sodium sulfide-treated insoluble membrane proteins migrated as a single homogeneous peak. Insoluble membrane proteins treated with selected detergents migrated chiefly as closely associated two-component systems. A heat-labile third component was observed following the major components. All solubilized insoluble membrane-proteins were examined in alkaline buffers to the isoelectric zone.

TABLE 4

SOLUBILITY CHARACTERISTICS OF THE SOLUBLE AND INSOLUBLE MEMBRANE PROTEINS

Solubilizing Agent	Soluble	Insoluble*
Water	+	-
Sulfuric acid (25%)	+	-
Sodium hydroxide (25%)	+	+
Urea (6 and 8 M)	+	-
Sodium lauryl sulfate		+
Dodecyl benzene sodium sulfonate		+
Thioglycolic acid		+
Sodium sulfide	+	+
Tergitol 7		+
Triton X-100		+

* Material was suspended in 0.02 M sodium chloride

TABLE 5

PHOSPHATASE AND XANTHINE OXIDASE ACTIVITY ASSOCIATED WITH SOLUBLE AND INSOLUBLE MEMBRANE PROTEINS

	Xanthine oxidase		Phosphatase	
	Sample[a] 1	Sample 2	Sample 1	Sample 2
	Units/mg. protein[b]			
Soluble	4.3	2.9	58	171
Insoluble	42	30	11	19
Membrane protein[c]	9		40	
	Units/ml.			
Cream (25% fat)[c]	143		400	
Skimmilk[c]	23		88	
Whey[c]	18		28	

[a] The data for samples 1 and 2 were supplied in a personal communication by Dr. C. A. Zittle.

[b] Zittle et al. units (1956b)

[c] These values were reported by Zittle et al. (1956b)

TABLE 6

CHEMICAL AND PHYSICAL PROPERTIES OF THE SOLUBLE AND INSOLUBLE FAT-MEMBRANE PROTEINS

	Soluble	Insoluble
Sulfur (%)	0.70	0.94
Nitrogen (%)	11.10	13.80
Phosphorus (%)	0.46	0.23
Ash (%)	7.06	2.08
Reduce Fehling's solution	+	-
Molish test	+	±
Nitroprusside test	-	+[a]
Color	White	Brown
Biuret test	+	+

[a] Heated momentarily to 75° C.

TABLE 7

COMPARISON OF THE AMINO ACID COMPOSITION OF THE FRACTIONATED MEMBRANE PROTEINS WITH LITERATURE VALUES

(All values calculated to 16 g. of N)

Constituent	Fat-membrane Protein				
	Soluble	Insoluble	Total Membrane Material[a]		
			A	B	C
Alanine	2.35	3.82	3.17	-	-
Arginine	5.48	8.22	7.02	6.99	5.0
Aspartic acid	5.41	7.60	6.63	-	-
Cystine	2.65	2.25	2.43	1.50	1.5
Glutamic acid	7.83	10.79	9.49	12.85	-
Glycine	2.34	3.88	3.20	3.76	3.0
Histidine	3.90	4.05	3.98	3.03	1.7
Isoleucine	4.35	4.58	4.48	5.67	3.5
Leucine	6.65	8.20	7.52	8.71	9.0
Lysine	7.33	8.60	8.02	5.92	6.1
Methionine	1.41	2.96	2.28	2.08	2.0
Serine	3.86	5.31	4.68	-	-
Threonine	4.49	5.31	4.95	6.03	6.4
Tryptophan	2.43	1.88	2.12	1.72	0.9
Tyrosine	4.64	4.40	4.50	-	-
Valine	3.60	6.70	5.42	5.65	5.4

[a] Legend: A = Values obtained when soluble fraction equals 44 and insoluble fraction equals 56 percent; B = Values reported by Brunner et al. (1953a); and C = Values reported by Hare et al. (1952)

TABLE 8

MOBILITY AND PEAK AREAS IN ELECTROPHORETIC PATTERNS OF THE FAT-MEMBRANE COMPONENTS AS SHOWN IN FIGURES II TO VI

Electrophoretic mobility patterns		Mobility			Area in percent		
		Peak No.			Peak No.		
Figure	Row	1	2	3	1	2	3
II	1	4.32	2.30		89.6	10.4	
	2	4.93	3.60		90.1	9.9	
	3	5.44	3.39		88.3	11.7	
	4	6.65	4.55		91.6	8.4	
	5	4.48	2.53		90.9	9.1	
	6	4.20			100.0		
III	1	5.50	4.16	2.07	40.4	48.9	10.7
	2	2.35	1.78	1.47	19.5	15.2	65.9
	3	3.86	3.22	3.09	6.0	20.8	73.2
	4	1.41	0.99	0.66	14.4	28.3	57.3
	5	0.95	0.54		48.0	51.7	
	6	3.21	2.30	1.21	11.0	63.8	25.2
	7	4.54	4.05		52.5	47.5	
	8	5.22	4.52	2.81	38.2	55.9	6.0
IV	1	3.88			100.0		
	2	6.81	6.01		3.5	96.5	
	3	6.11	5.75		16.1	83.9	
	4	9.15	7.12		8.1	91.9	
V	1	9.80	9.33	8.10	32.5	52.9	14.6
	2	9.52	7.85		32.4	67.6	
	3	11.63	9.05	6.79	11.8	83.6	4.6
	4	10.20	8.82	6.87	41.7	48.7	9.6
VI	1	6.19	4.93		6.9	93.1	
	2	5.54	5.22		36.5	63.5	
	3	5.85	5.41		14.1	85.9	

TABLE 9

SPECTOGRAPHIC ANALYSIS OF THE ASH
OF THE MEMBRANE PROTEINS

Element	Soluble	Insoluble
Aluminum	Trace	Trace
Calcium (%)	0.503	0.319
Copper (p.p.m.)	132	82
Iron (p.p.m.)	91	510
Magnesium (%)	0.548	0.227
Manganese (p.p.m.)	11	11
Molybdenum (p.p.m.)	5	125
Phosphorus (%)	0.416	0.289
Silver	Trace	Trace
Zinc (p.p.m.)	17	22

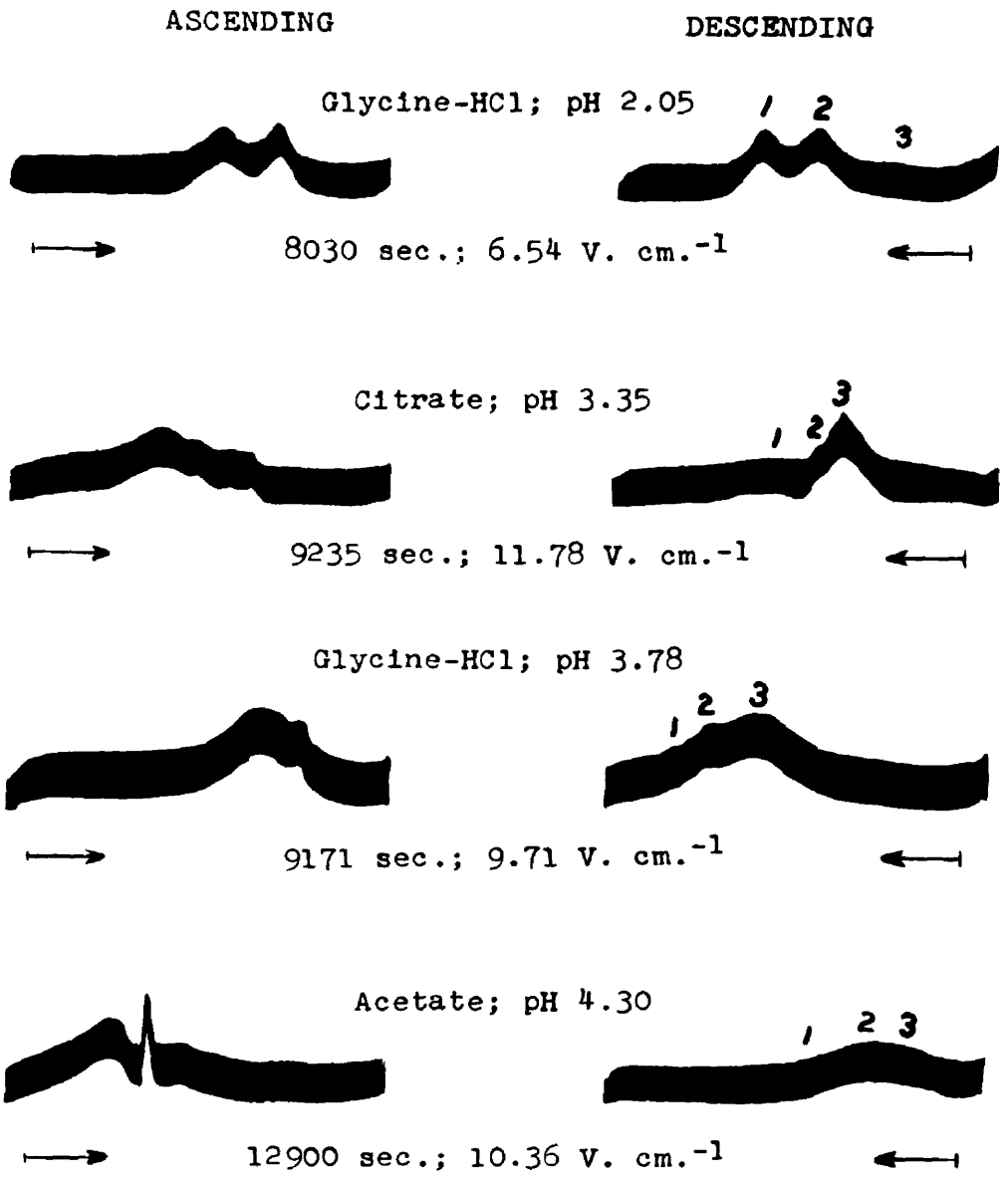


FIGURE III. Electrophoretic mobility patterns of soluble membrane proteins in various buffers.

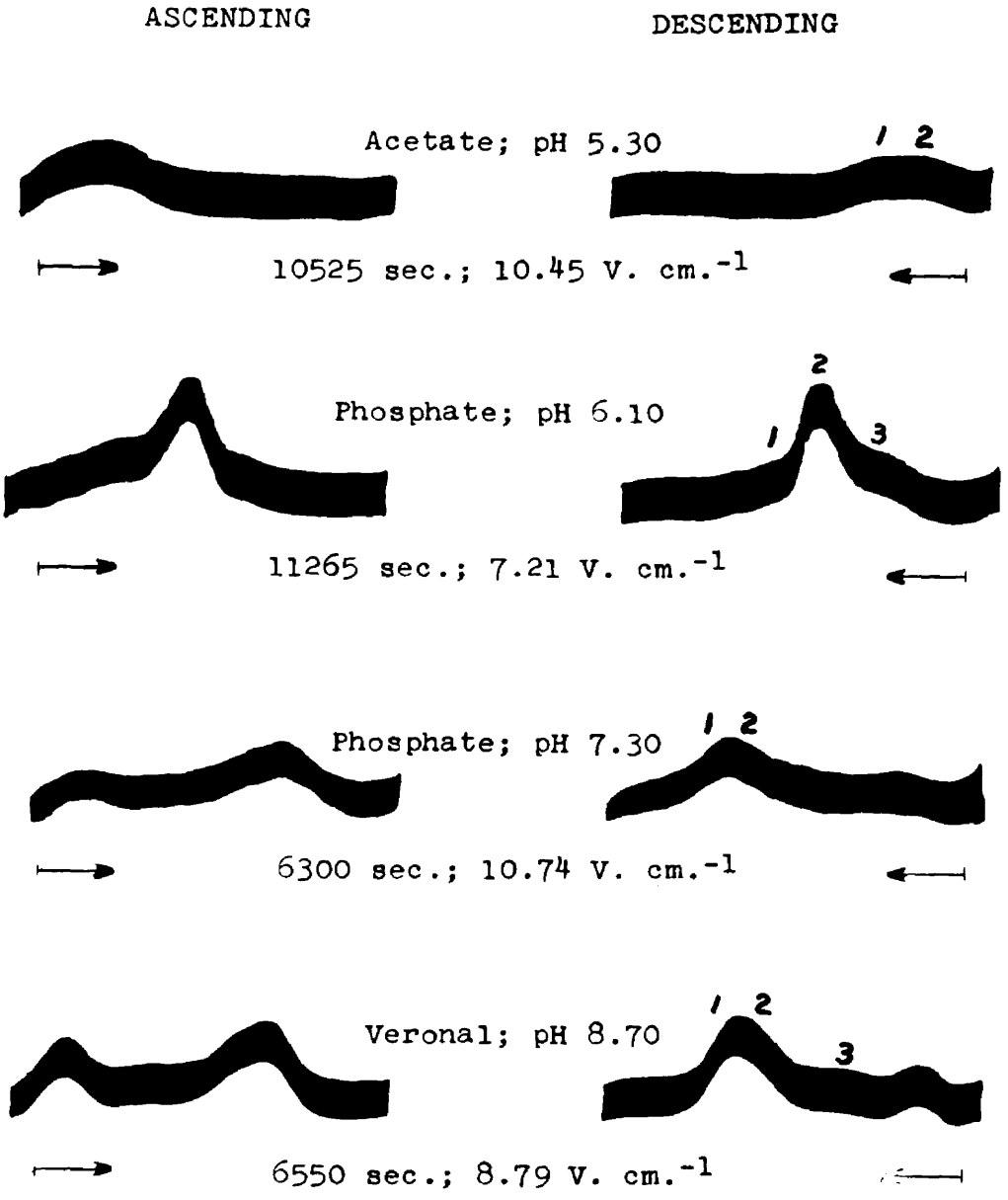


FIGURE III. (Continued) Electrophoretic mobility patterns of soluble membrane proteins in various buffers.

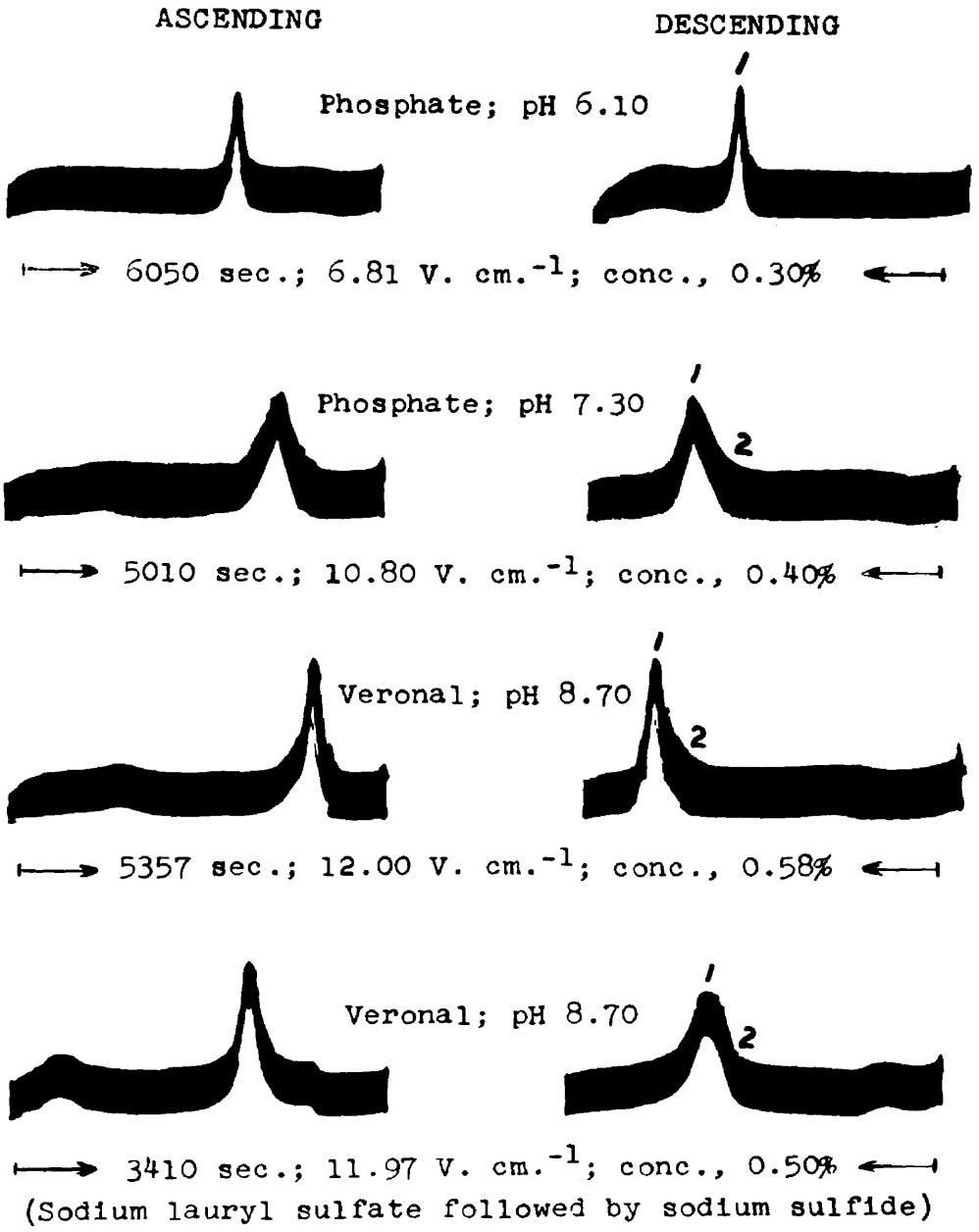


FIGURE IV. Electrophoretic mobility patterns of insoluble membrane proteins solubilized with sodium sulfide.

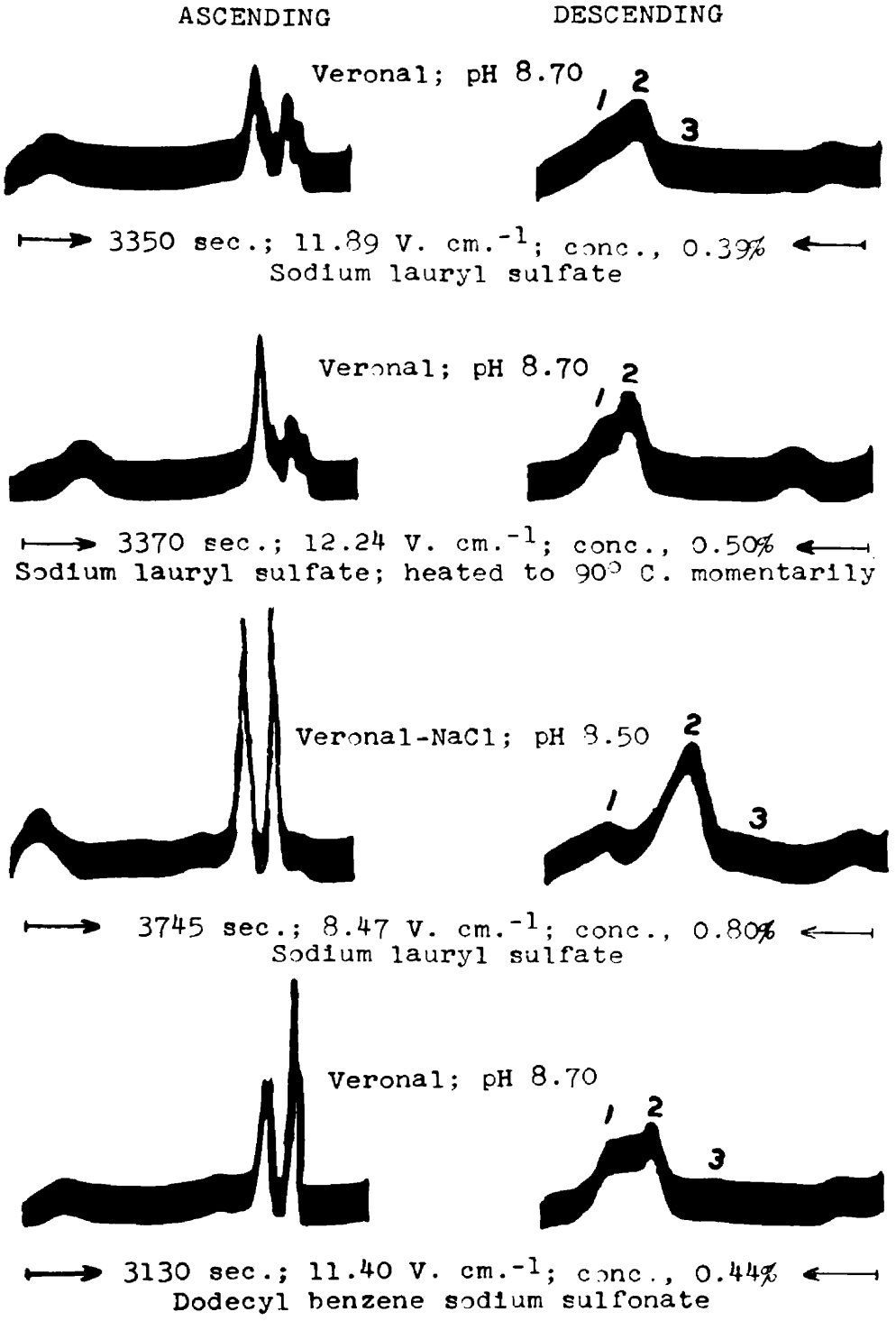


FIGURE V. Electrophoretic mobility patterns of insoluble membrane proteins solubilized with detergents.

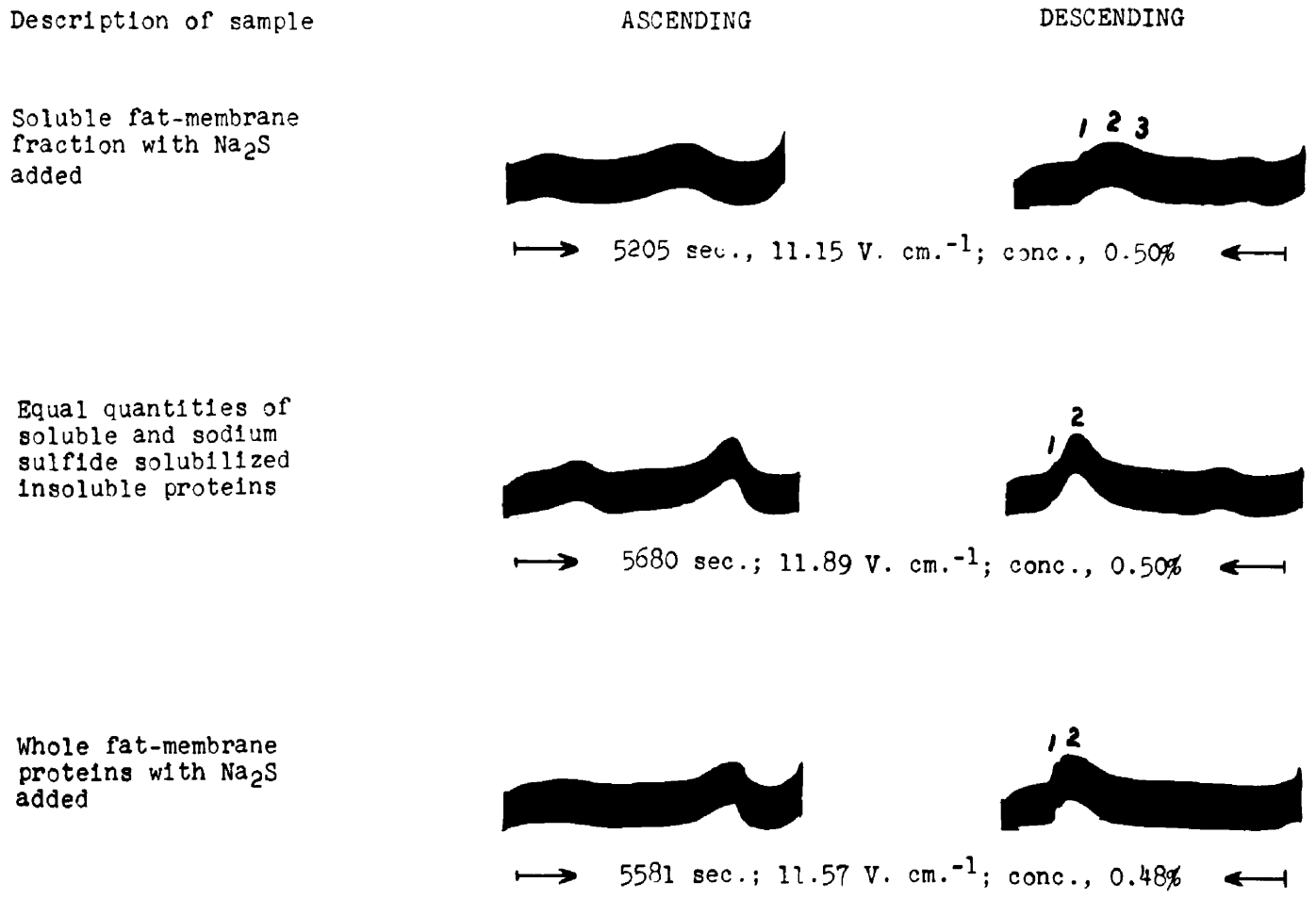


FIGURE VI. Electrophoretic mobility patterns of Na_2S-treated fractions of the fat-membrane in veronal buffer at pH 8.70

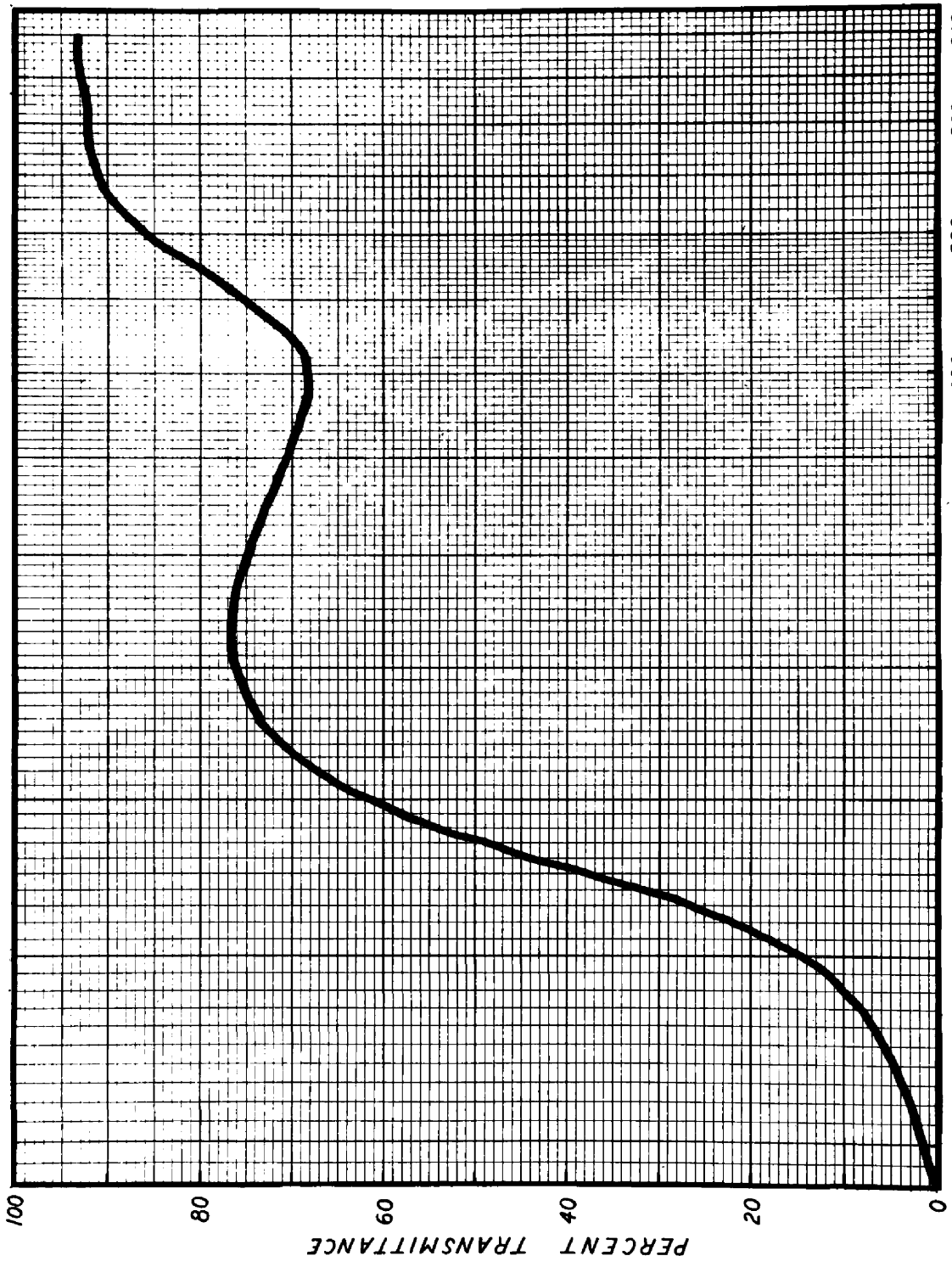


FIGURE VII. Ultraviolet spectrogram of the soluble membrane proteins.

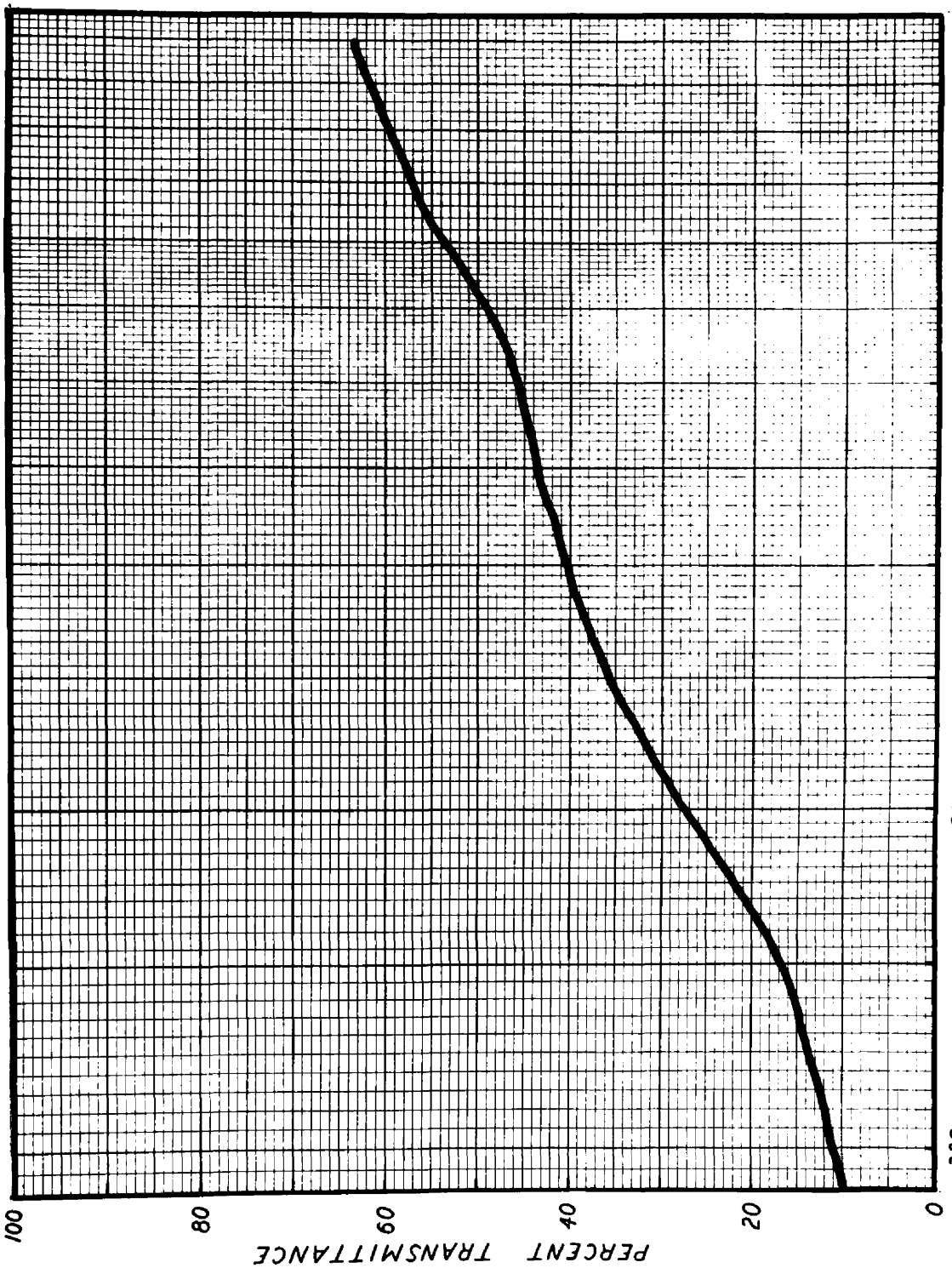


FIGURE VIII. Ultraviolet spectrogram of the insoluble membrane proteins.

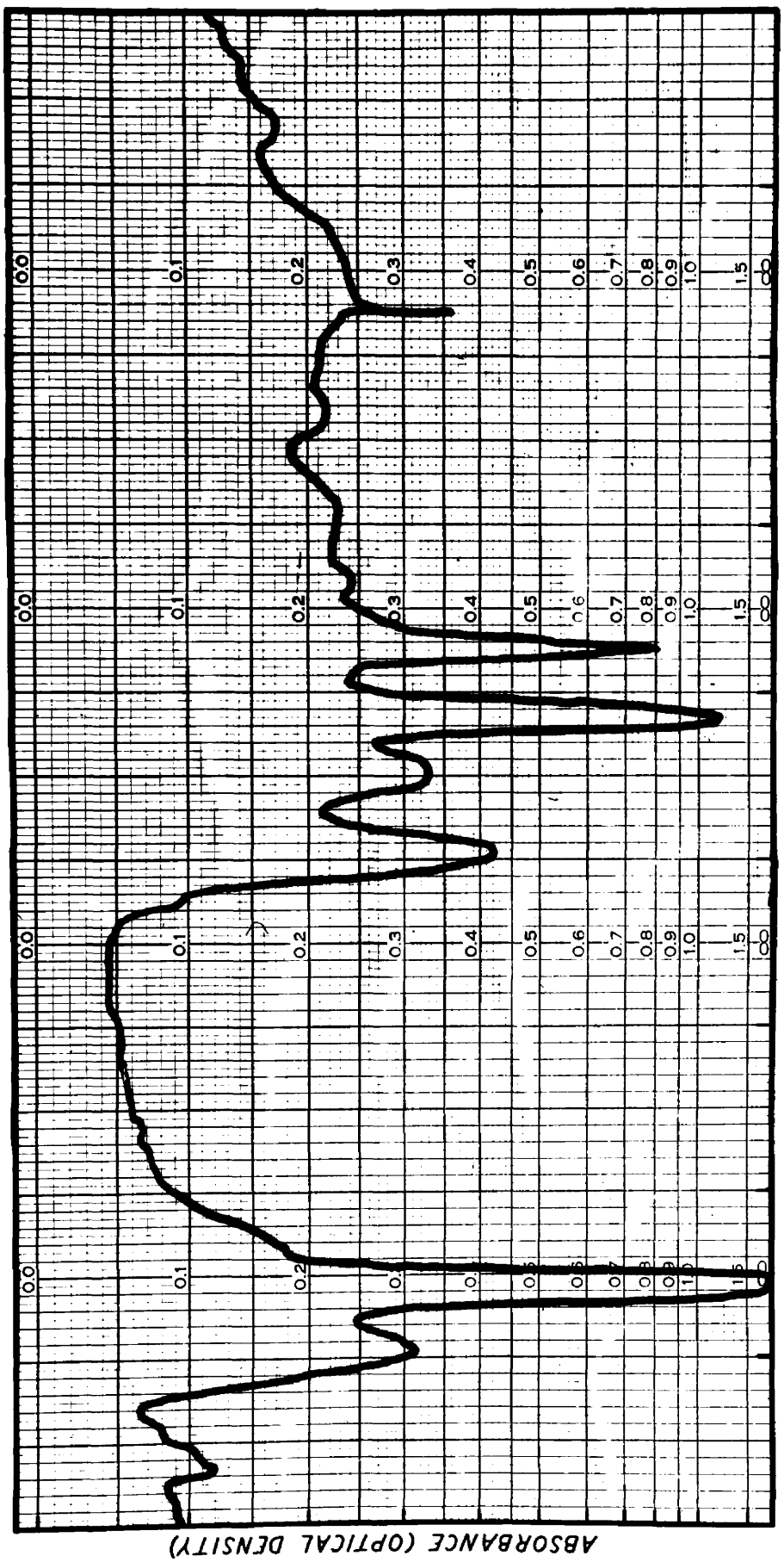


FIGURE IX. Infrared spectrogram representative of the soluble and insoluble membrane proteins.

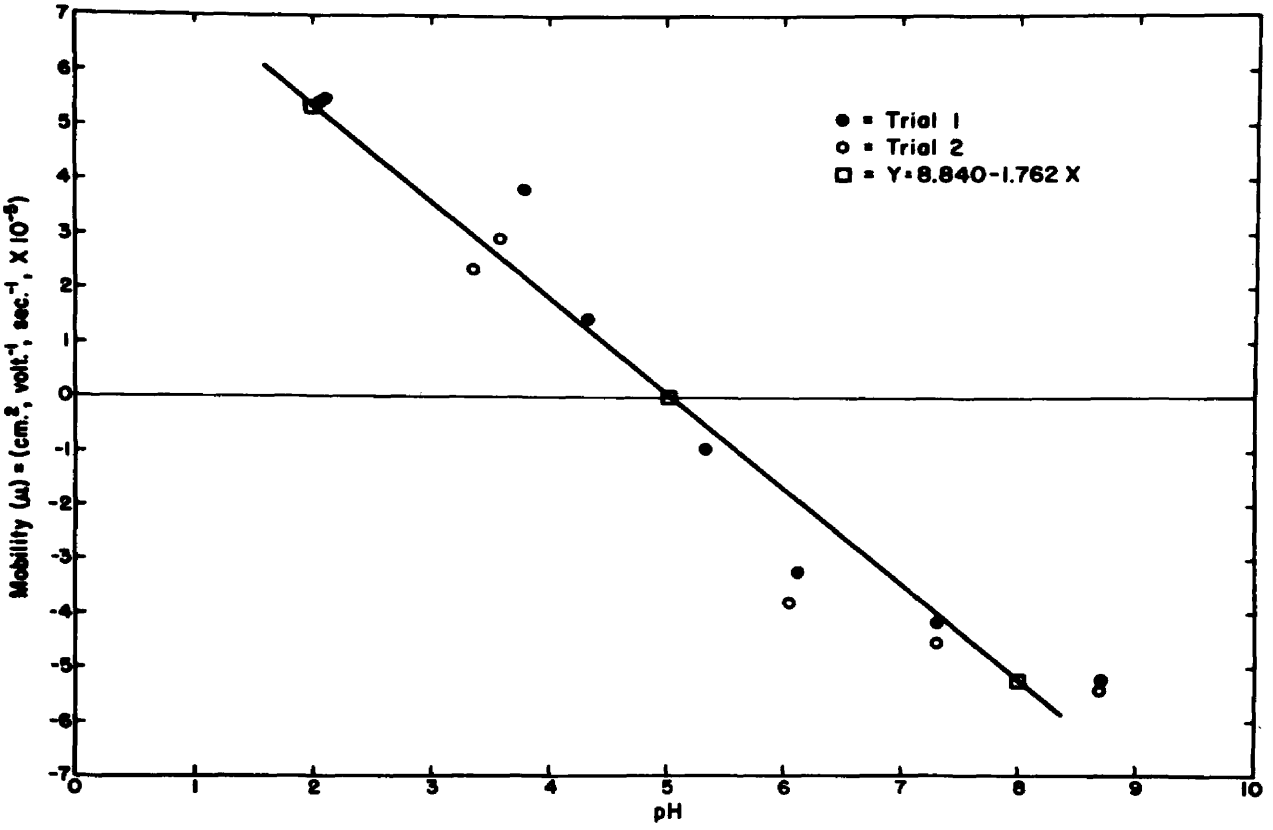


FIGURE X. pH-mobility curve for the leading component of the soluble fat-membrane proteins.

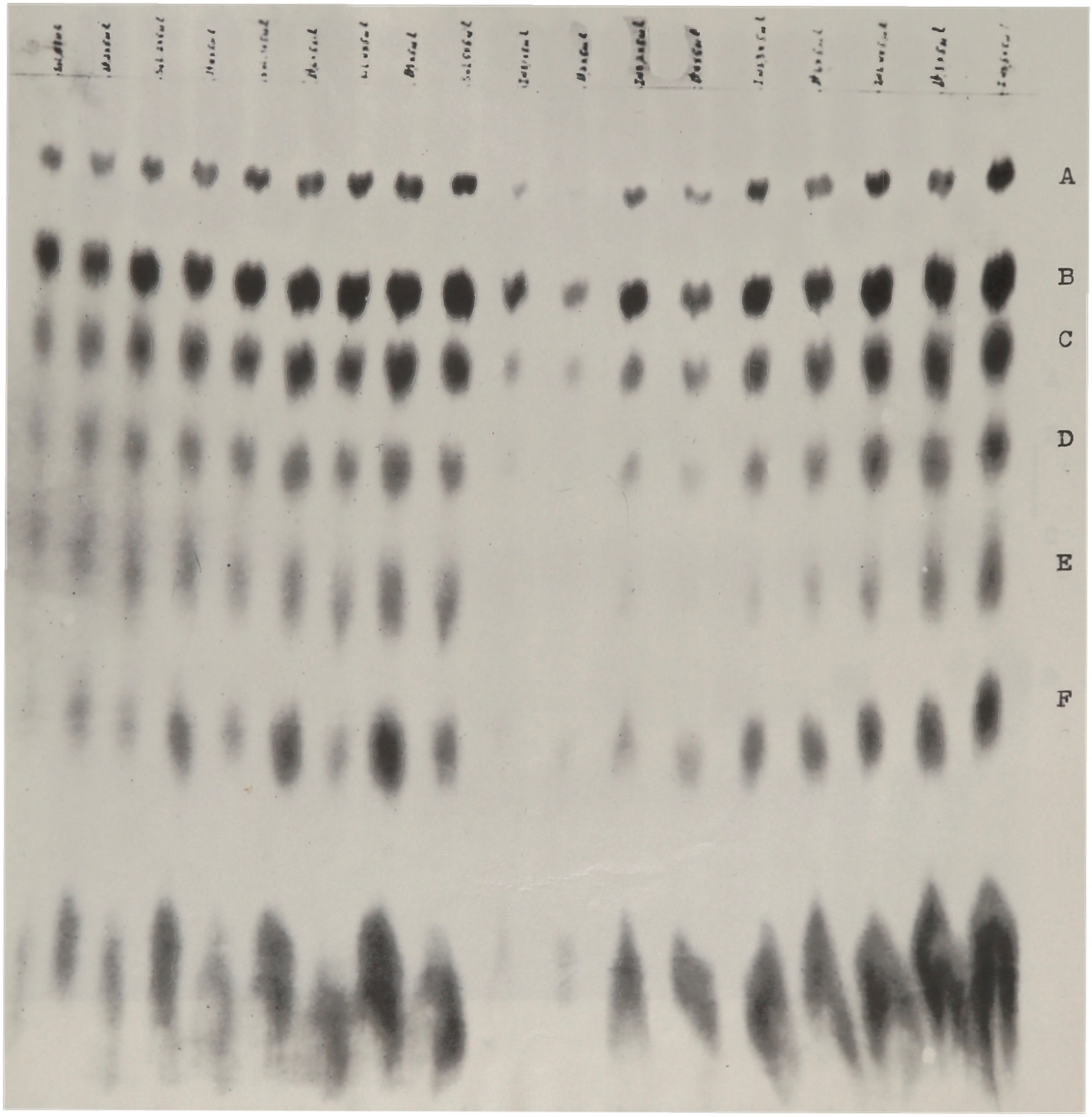


FIGURE XI. Quantitative amino acid chromatogram showing: A-aspartic acid, B-glutamic acid, C-serine, D-glycine, E-threonine, and F-alanine.

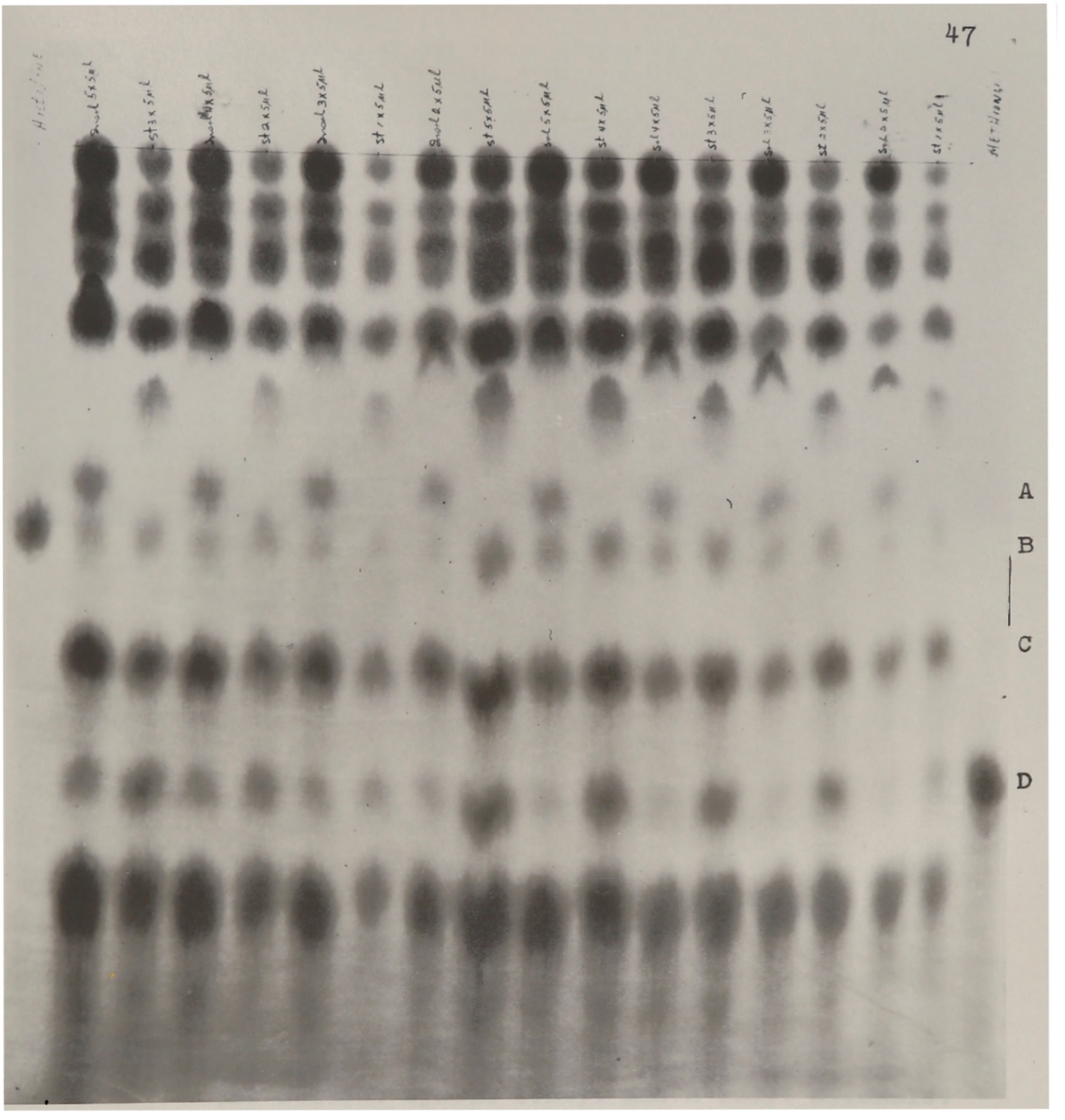
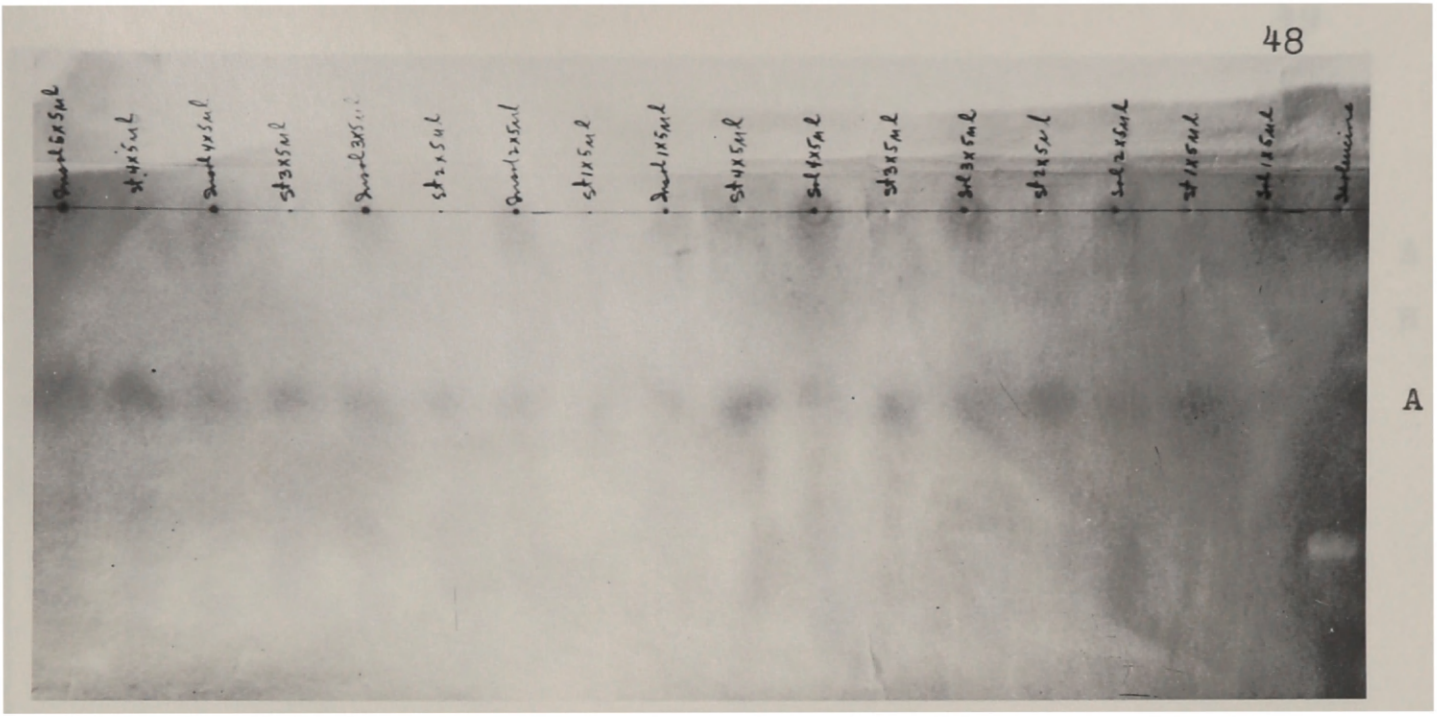


FIGURE XII. Quantitative amino acid chromatogram showing: A-tyrosine, B-histidine, C-valine, and D-methionine.



A

FIGURE XIII. Quantitative amino acid chromatogram showing: A-proline. Top-portion of chromatogram from the solvent system benzyl and n-butyl alcohols. After development with isatin the spots were greenish-blue against a yellow background.

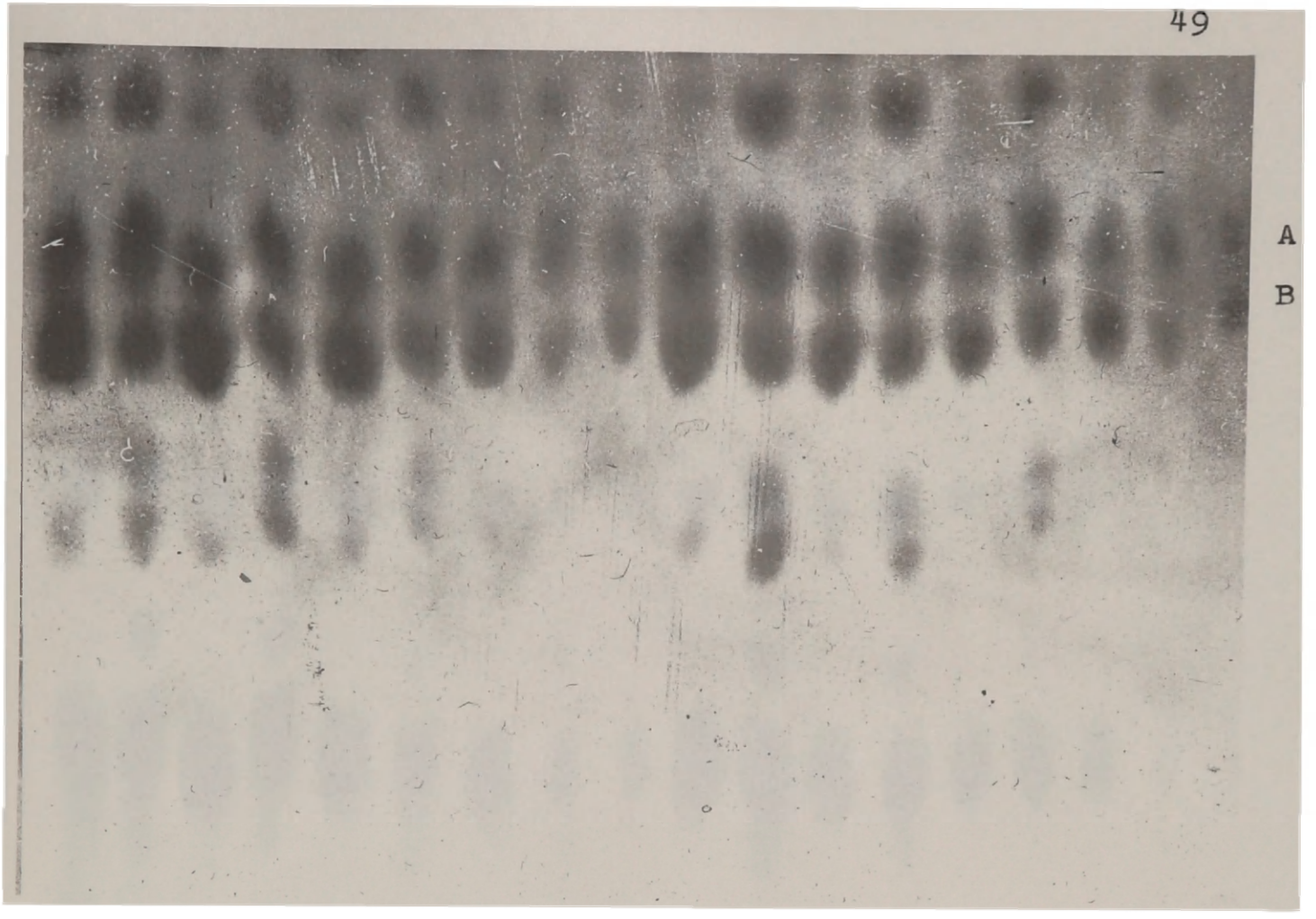


FIGURE XIV. Quantitative amino acid chromatogram showing: A-isoleucine, and B-leucine. Lower portion of chromatogram from the solvent system benzyl and n-butyl alcohols.

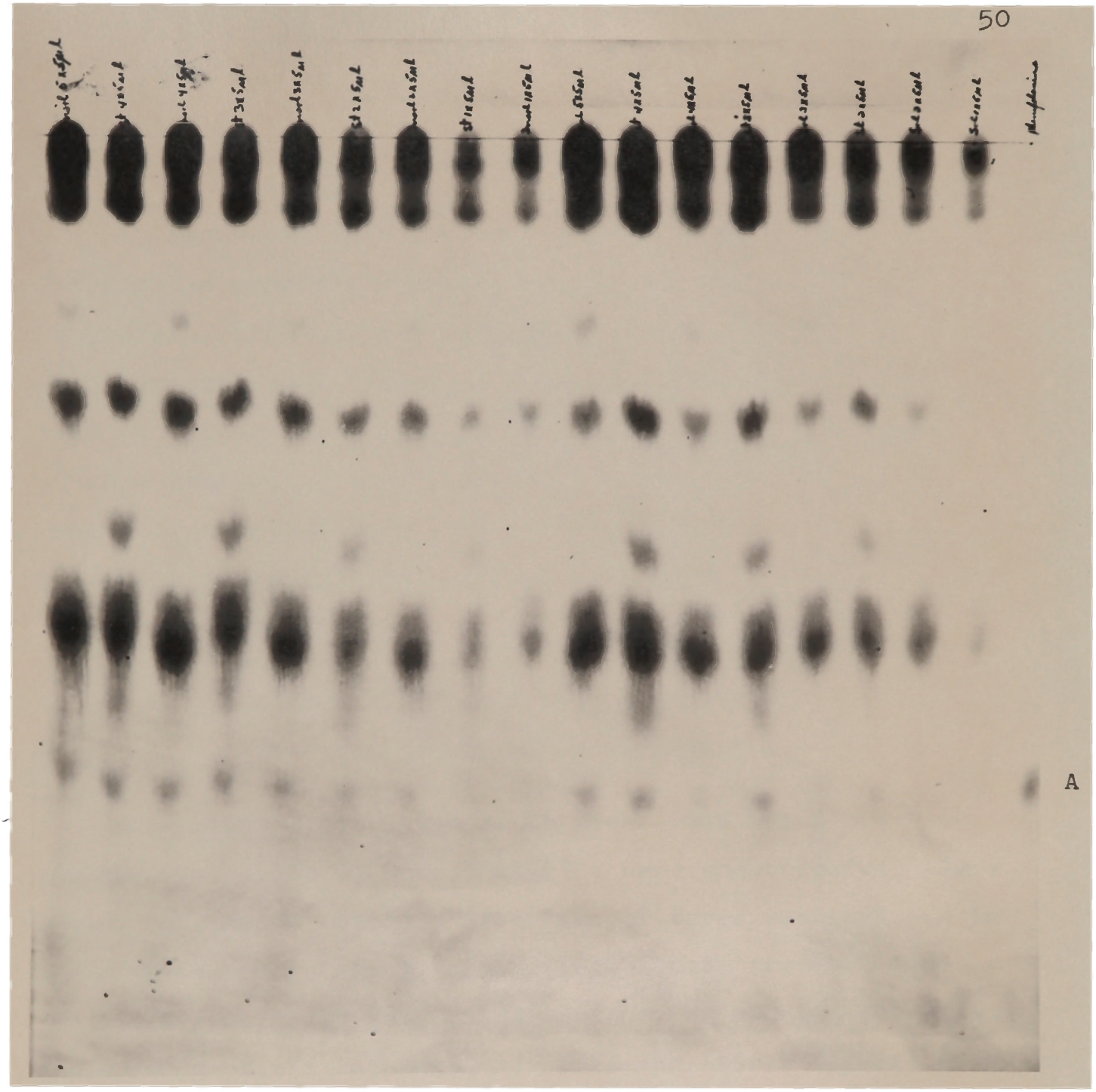


FIGURE XV. Quantitative amino acid chromatogram showing:
A-phenylalanine.

DISCUSSION

"Lipoprotein" has frequently been used to describe the fat-globule membrane complex. Since the membrane is composed of protein, high-melting triglycerides, and phospholipides, this term has justification. Hence, in the following discussion, lipoprotein will be used synonomously with the fat-globule membrane. The isolated lipoprotein in this study does not represent the intact membrane; however, the isolated materials represent the membrane most closely associated with the fat-globules.

Effect of Various Procedures Used to Isolate the Membrane Proteins

Raw materials. Attempts to wash cream which had been stored (for about 24 hours) at 5°C. were unsuccessful. Aged cream "oiled-off" rapidly after two or three washings and in some cases completely de-emulsified. Cream prepared from fresh, raw milk did not oil-off during the washing procedure consisting of six washings. Jenness and Palmer (1945a) emphasized the importance of using fresh milk in preparing washed cream. These investigators isolated 0.66 grams protein and 10.8 milligrams of phosphorus per 100 grams of fat from washed cream prepared from uncooled raw milk. Washed cream prepared from milk aged for 24 hours at 3.3°C. only yielded 0.42 grams of protein and 9.7 milligrams of phosphorus per 100 grams of fat. These data can be interpreted to show that

a portion of the phosphorus was concentrated closer to the fat globules than to the proteins. The phospholipides of the membrane would be a logical source of phosphorus.

Viscous, stable emulsions frequently formed whenever washed cream was churned in a conventional laboratory churn. Washed cream was de-emulsified most rapidly in a horizontally agitated gallon jar.

Thirty gallons of milk yielded 4,116 grams butterfat, and 4,306 grams of serum of pH 7.0 containing 1.22 percent and 0.78 percent total solids and fat, respectively. Thus, 1.27 grams of membrane material per 100 grams of butterfat were recovered. Since the membrane was found to contain about 40 percent protein, the recovery of protein can be estimated at about 0.51 grams per 100 grams of fat. Jenness and Palmer (1945a) reported a protein recovery ranging from 0.46 to 0.86 grams per 100 grams of fat.

Concentration of membrane materials. Approximately 16 to 20 hours were required to condense serum according to the method of Brunner et al. (1953a). During the condensing procedure, the serum foamed considerably and a large portion of membrane material adhered to the condensing flask. According to Neurath and Bull (1938), surface-film formation and adsorption at interfaces were conducive to the denaturation of proteins. Moreover, the condensed mixture froze when cooled to about 0°C., and cold ethanol combined with the material to form an unwieldy emulsion.

Slowly freezing the serum to concentrate the membrane material did not concentrate the solids in a satisfactory

manner. Isoelectric-precipitated membrane material subsequently pretreated with ethanol and extracted with ether resisted solution. According to Lisse (1940), proteins at the isoelectric point are most sensitive to alcohol. Further, the supernatant from the isoelectric-precipitated material contained a yellowish-green fluorescent material which suggests that a form of riboflavin might be associated with the membrane proteins. Ball (1939) reported that flavin adenine dinucleotide was closely associated with membrane proteins.

Membrane material was readily concentrated from 32 to 34 percent solids upon adjusting the serum to 55 percent ammonium sulfate concentration and subsequent centrifugation. The supernatant was biuret-negative indicating a complete removal of proteins. Membrane material was not removed efficiently from the serum whenever a 50 percent ammonium sulfate concentrate was used. The concentrated membrane material had a reddish-brown color. When the ammonium sulfate precipitate was held at 0 to -5°C. and treated with cold ethanol and ether, no hard masses or emulsions were formed. Alcohol was readily removed from the mixture when it was filtered and washed several times with cold ether in a -20°C. cold room. The residue on the filter paper had a friable appearance following the cold ether extractions.

Effect of various organic solvents. The data in Table 2 show that membrane proteins pretreated with 35 percent ethanol had the highest nitrogen content and appeared to be the most soluble. It is also apparent that cold ethanol pretreatments were more effective than cold n-butanol treat-

ments in facilitating lipid removal. Furthermore, the data reveal that cold ethanol was as efficient as room temperature n-butanol in achieving the removal of lipids from the membrane material (Table 3). According to Morton (1950) and Zittle and DellaMonica (1952), n-butanol had specific ability to induce the separation of lipoprotein complexes. Zittle et al. (1956b) used n-butanol to dissociate the fat-globule membrane material and the protein preparation contained 11.8 percent nitrogen. This value is about one-half percent lower than reported values (Weise and Palmer, 1934; Hare et al., 1952; and Brunner et al., 1953a). The low nitrogen values obtained from n-butanol-treated membrane-proteins can be assumed to be due to an incomplete removal of lipids. Furthermore, the practice of using room temperature n-butanol on protein materials is open to question. Leading authorities, such as Cohn (1943) and Taylor (1955), emphasize the importance of low temperatures whenever organic solvents are in contact with proteins. On the basis of the above discussion, ethanol was selected as a pretreatment technique prior to ethyl ether extractions for the removal of lipid from the fat-globule membrane.

In the realm of blood chemistry, cold ethanol has frequently been employed to disrupt lipoprotein complexes (Hardy and Gardiner, 1910; McFarlane, 1942; Cohn, Strong, Hughes, Mulford, Melin, and Taylor, 1946; and Jergensons, 1955). In this study, lipid materials were not removed by McFarlane's (1942) freezing-thawing technique. Apparently the lipid of the fat-membrane is more strongly complexed than

the lipids of human serum.

During the preliminary investigations, the membrane proteins were decolorized frequently during the ether extractions. This presumably was due to the presence of peroxide groups in the ether. Moreover, when the ethanol was increased above 35 percent, the intensity of the brown color diminished and sometimes disappeared. The loss of the brown color was accompanied by a loss of xanthine oxidase activity.

When 50 grams of concentrated lipoprotein were added to 100 milliliters of a 35 percent ethanol-ether solution, the final concentration was about 26 percent ethanol.

Chemical, Physical and Biological Characteristics of the Fat-globule Membrane Proteins

Electrophoretic characteristics. The electrophoretic mobility patterns presented in Figure II were selected from some of the exploratory runs. These patterns showed qualitative similarities when treated with 30 to 95 percent ethanol. Quite similar patterns were obtained for n-butanol-treated membrane except for a greater degree of diffusion. Throughout the investigation, it was noted that the optical clarity of the protein solution was easier to achieve with the ethanol pretreated than with the n-butanol pretreated membrane proteins. The skewed shape of these patterns indicated a heterogeneous protein system. An inspection of the electrophoretic data in Table 8 reveals that the mobility of the major migrating peak was fastest in the Miller and Golder

(1950) buffer, which ranged from pH 2 to 12 and sodium chloride supplied 90 percent of the ionic strength. In runs prepared with this buffer system, the false boundaries moved abnormally in the direction of migration. This is apparent in Figure 2, row 4.

Delta and epsilon boundaries were first thought to be due to slowly migrating protein or carbohydrate constituents. Longsworth and MacInnes (1940) explained the presence of false boundaries. When an electrical current is passed through a Tiselius cell, causing the descending boundary to move, the composition of the protein-containing buffer becomes changed in respect to the original buffer. Thus, an epsilon boundary is formed between two solutions of the same buffer, each with a different concentration. The delta boundary on the ascending side involved a gradient of protein concentration in addition to salt concentration gradient. Thus, the delta boundary is somewhat larger than the epsilon boundary. Buffers which contain large amounts of a neutral salt are frequently used in the electrophoresis of blood serum, whereas milk proteins have been investigated generally in the more conventional types of buffers.

Tap water-extracted membrane-protein appeared to be identical with the protein extracted with buffer solution. This is illustrated by the electrophoretic patterns in Figure II, row 1. When the unfractionated fat-globule membrane proteins were dispersed in a buffer solution and centrifuged before electrophoresis, a brownish-red insoluble pellicle was found in the bottom of the centrifuge tube. Thus, it was

suspected that the proteins in solution were not representative of the fat-membrane proteins.

In the veronal buffer at pH 8.8, the soluble membrane proteins pretreated with 35 percent ethanol were most mobile. The proteins prepared with room temperature n-butanol had the lowest mobility. Since a decrease in mobility is indicative of changes in the protein, the assumption can be made that room temperature n-butanol produced undesirable changes in the protein system.

The electrophoretic mobility patterns in Figure III represent membrane proteins soluble in 0.02 M. sodium chloride solution. Various degrees of resolution were observed on either side of the isoelectric zone. On the acid side, two and three components were resolved; whereas, on the alkaline side only one major skewed peak predominated. Apparently the soluble protein polymerized into various sized aggregates dependent on the pH of the buffer system. Thus, the apparently heterogeneous system on the acid side might have polymerized into a more homogeneous unit on the alkaline side. At pH 5.30, a considerable amount of protein precipitated during dialysis and low mobilities were observed for the components in the supernatant, indicating that the isoelectric area for the soluble membrane proteins was about pH 5.30. The fact that the mobilities were almost identical for the leading component of the diagrams in Figure II, row 4 and Figure III, row 8 indicated that lyophilization did not affect the soluble membrane proteins. The electrophoretic mobility patterns in Figure III approximate the patterns presented by Brunner et al. (1953c).

The electrophoretic mobility patterns shown in Figure IV represent insoluble membrane-proteins treated with sodium sulfide. More protein precipitated from the buffer solution as the pH was lowered. Attempts to use acidic buffers resulted in the total precipitation of protein. Similar precipitations were encountered with proteins solubilized with detergents. When a solubilizing concentration of sodium sulfide was added to the proteins suspended in saline solution, the pH was 11.6. Accordingly, all electrophoretic work for the insoluble membrane proteins was carried out in alkaline buffers. The mobility patterns indicate that sodium sulfide reduced the insoluble proteins to a homogeneous mixture. This is illustrated further in Figure IV, row 4, representing electrophoretic patterns of insoluble proteins solubilized with sodium lauryl sulfate and then treated with sodium sulfide.

The effect of sodium lauryl sulfate and dodecyl benzene sodium sulfonate on the insoluble membrane-proteins is shown in Figure V With the exception of patterns in row 2, each run revealed two main components and a third, slower migrating, minor component. The minor component was removed by heating momentarily to 90°C. and is illustrated by the electrophoretic patterns in row 2. The heat-labile minor component might have been xanthine oxidase since this enzyme is readily inactivated at 75°C. for five minutes (Zittle et al., 1956a). In row 3, sodium lauryl sulfate-solubilized material was examined in the Miller and Golder (1950) veronal-sodium chloride buffer. The results were quite similar to the patterns in rows 1 and 2, particularly on the descending side. Interestingly, the

false boundaries did not move abnormally as in Figure II, row 4, which indicates that the nature of the proteins affects the movement of the false boundary anomalies.

The question arises as to whether the areas under the peaks are due to proteins or to detergent materials. However, experimental work revealed that sodium lauryl sulfate precipitated when cooled to 0°C. Thus, the possibility was excluded that any of the peaks were due to sodium lauryl sulfate. In a veronal buffer of pH 8.6 and 0.10 ionic strength, Foster (1949) reported a mobility of 18-19 x 10^{-5} $cm.^2$ $volt^{-1}$ $sec.^{-1}$ for dodecyl benzene sodium sulfonate. This is almost double the mobility values reported for the solubilized proteins in Figure V, row 4. Again, the possibility was excluded that a peak area represented dodecyl benzene sodium sulfonate. The number two peak (ascending side) in the sample treated with dodecyl benzene sodium sulfonate seemed inclined to split into two components.

The possibility of a detergent-protein complex was indicated in this study. First, an optimum detergent-protein ratio was needed to effect solubility. Second, the proteins did not precipitate in an alkaline medium during dialysis. The effect of sodium sulfide on the soluble proteins is shown in Figure VI, row 1. The patterns were diffused which indicated that the proteins were broken into small units. Row 2 represents patterns obtained from a mixture of equal quantities of the dialyzed soluble proteins and sodium sulfide-solubilized insoluble proteins. Interestingly, the electrophoretic patterns were unlike those for either the soluble proteins or solubilized insoluble proteins. However, the

mobility of the complex was similar to the leading component of the soluble proteins in a similar buffer. Stanley, Whitnah and Andrews (1951) suggested the possibility of interactions between various milk components. The diagrams in row 3 were similar to those of row 2. Again, the absence of the characteristic spike for sodium sulfide-treated insoluble proteins was evident.

Isoelectric zone for soluble proteins. Since it was not possible to identify the same component in all of the electrophoretic patterns, the mobility of each component was selected for ordinate values in a pH-mobility plot. Two separate trials were considered in these calculations. Seven electrophoretic runs were made in the first trial and six in the second. Using the method of curvilinear regression, these points did not differ significantly from a straight line at the probability level of five percent, therefore the 13 runs were combined. The combined runs were also linear at the five percent probability level. The regression equation for the combined data is $Y = 8.840 - 1.762X$ where Y represents mobility and X denotes pH. X is equal to 5.02 when Y is equal to 0. Thus, zero mobility for the leading component was at pH 5.02. The original data for the leading component and its regression curve are shown in Figure X. The same statistical method was used to derive a regression equation for components two and three. For component two, the regression equation was $Y = 7.250 - 1.455X$. Thus, the point of zero mobility for component two was at pH 4.98. The third component had a regression equation of $Y = 4.663 - 0.900X$. The isoelectric

point for component three was then fixed at pH 5.18. Palmer and Weise (1933) and Jack and Dahle (1937a) reported the isoelectric zone of membrane proteins to be at about pH 3.8 and 4.3, respectively. These workers reported values for the unfractionated protein; therefore, their data should not be compared with the above results.

Solubility studies. The insolubility of at least a portion of the fat-membrane proteins has been suggested by several workers (Hattori, 1925; Titus, Sommer and Hart, 1928; and Palmer and Weise, 1933). According to Titus, Sommer and Hart (1928), the membrane proteins were quite similar to casein. They believed that their preparation was contaminated with some unknown substance since the isolated protein would not dissolve in 0.5 N sodium hydroxide. Current knowledge suggests that these researchers isolated casein contaminated with membrane proteins. Pedersen (1936) reported an insoluble material in separator slime which was assumed to be related to casein. A more plausible explanation would seem to indicate that the insoluble material was from the fat-globule membrane.

The data in Table 4 illustrate the solubility characteristics of the membrane proteins. The soluble fraction was readily soluble in aqueous solvents and was readily salted-out by half-saturation with ammonium sulfate. According to the classical protein classification systems, this indicates that the soluble fraction was "globulin" in nature. Sodium sulfide and thioglycolic acid readily solubilized the insoluble proteins. Since thioglycolic acid emitted noxious odors, this compound was not used further in the solubility studies.

The fact that these compounds were effective as solubilizing agents suggests that the insoluble proteins have a disulfide linkage.

Detergent-type chemicals have been found useful as de-emulsifying agents in the preparation of butterfat (Patton, 1952; and Stine and Patton, 1952). Patton (1952) suggested that the lowering of interfacial tension probably facilitated the release of milk fat from the globules of milk. While this is probably a safe assumption, the possibility cannot be overlooked of a soluble complex formation between the de-emulsifying agent and the insoluble membrane proteins. Thus, as the solubilized membrane proteins go into solution, the fat is freed. Stine and Patton (1952) reported that "of twenty-six agents which de-emulsified cream quantitatively, twenty-four were of the cationic type, the other two being nonionic." However, King (1955), in citing the work of Stine and Patton (1952), referred to twenty-four anionics and two nonionics in discussing de-emulsifying agents. The surface-active agents used in this study were anionic with the exception of Triton X-100 which was nonionic. It is of interest to note that Foster (1949) used dodecyl benzene sodium sulfonate to solubilize zein prior to electrophoretic and sedimentation velocity studies.

Whenever sodium lauryl sulfate or dodecyl benzene sodium sulfonate was added to turbid aqueous protein suspensions, optical clarity was achieved in a few minutes. Insoluble proteins heated in the presence of Triton X-100 remained turbid until cooled to 62°C. Turbidity was again readily induced

by heating the mixture. This phenomenon indicated that the Triton X-100 protein complex had a negative critical solution temperature. Furthermore, proteins solubilized with Triton X-100 and Tergitol 7 were viscous and thus were rejected for electrophoretic studies. In addition, Tergitol 7 solutions became turbid upon cooling. Lyophilized insoluble membrane proteins did not yield to solubilization treatments. This held in the case of detergent-like chemicals and sodium sulfide.

The insoluble membrane proteins formed a stable suspension whenever these proteins were dispersed in aqueous solvents; however, the proteins were readily precipitated upon freezing and thawing. Apparently, the insoluble membrane proteins are sensitive to freezing temperatures.

Enzyme studies. Enzyme activity was employed in this study as an indicator of undesirable changes in the protein system. Although this purpose was served by this criterion, other interesting observations were made. Table 5 reveals that phosphatase was concentrated in the soluble fraction whereas xanthine oxidase was concentrated in the insoluble fraction. Zittle et al. (1956b) reported that freeze-drying caused a 15 and 46 percent loss of alkaline phosphatase and xanthine oxidase, respectively. Their data showed 40 units per milligram of phosphatase activity and 9 units per milligram of xanthine oxidase activity on a dry-protein basis. When corrected for loss in enzymatic activity due to freeze-drying, the samples used in this study were found to contain 49 and 145 units of phosphatase activity per milligram in the soluble portion, and 16 and 23 units of xanthine oxidase

activity per milligram in the insoluble fraction. The fact that the phosphatase activity followed the soluble portion and xanthine oxidase activity was concentrated in the insoluble protein was of interest. This behavior indicated that the cold ethanol-ether treatments were specific in releasing phosphatase from the lipoprotein complex. This indicates that n-butanol is not unique in its ability to disrupt lipoprotein complexes.

Morton (1953, 1954) concluded that the enzymatic activity of the fat-globule membrane was associated with microsomes adsorbed on the protein layer surrounding the fat globules, but this conclusion is not entirely supported by the data of other workers. Jenness and Palmer (1945a) found that the phospholipide/protein ratio in butter serum was higher than in washed cream buttermilk. This indicated that phospholipides were concentrated at the fat membrane interface. Furthermore, Zittle et al. (1956b) measured the xanthine oxidase and alkaline phosphatase activity in a series of washed creams. The ratio of xanthine oxidase to alkaline phosphatase was 1:1.8, 1:2.18, 1:2.18, and 1:3.3 in four samples of washed cream, respectively. If xanthine oxidase and alkaline phosphatase were associated with a unit particle, one would expect the enzyme ratios in washed cream to remain constant during the washing procedure. Ball (1939) showed that the xanthine oxidase content of skimmilk increased as the milk aged, especially at low temperatures. Polonovski, Baudu and Neuzil (1949) pointed out that freezing or the action of surface-active agents released xanthine oxidase from the membrane

protein into true solution. In addition, these authors showed that a portion of the enzyme resisted the means of division employed and remained intact on the membrane. From this discussion it seems clear that a more complete study is needed in this area of research.

Chemical composition. The chemical composition of the fractionated membrane proteins was different than values reported for the entire protein. The values in Table 6 can be approximated to whole protein when the values 44 and 56 percent are used for the soluble and insoluble portions, respectively. On this basis, the whole protein contained 0.92 percent sulfur. Weise and Palmer (1934) reported 0.96 percent sulfur. Hare et al. (1952) found 1.68 percent sulfur; however, this was not consistent with their values reported for the sulfur-containing amino acids. Their amino acid data accounted for only 0.64 percent sulfur. In this study, the amount of sulfur in the soluble portion calculated from the methionine and cystine data coincide exactly with the chemically determined sulfur. Methionine and cystine in the insoluble fraction accounted for 1.08 percent sulfur as compared to 1.03 percent sulfur determined chemically. That this fraction probably contained cysteine was indicated by its positive sulfhydryl reaction.

The protein nitrogen values for the unfractionated membrane ranged from 12.2 to 12.6 percent. The former value is more consistent with values reported previously (Weise and Palmer, 1934; Hare et al., 1952; and Brunner et al., 1953a). Since a portion of the proteins are water soluble, it is not

surprising that variable nitrogen values have been found. Whenever the membrane proteins were fractionated, the soluble portion always had a lower nitrogen content than the insoluble portion. Although this trend was invariant, the nitrogen values obtained varied among preparations for the two fractions. For the soluble fraction, nitrogen values ranged from 9.5 to 11.5 percent and from 12.9 to 13.9 percent for the insoluble fraction. A representative experiment is reported in Table 6. The insoluble fraction approached the nitrogen content of a simple protein.

Insoluble proteins prepared from lyophilized whole protein had a higher nitrogen value than that prepared from non-lyophilized protein. During the experiments, some of the insoluble proteins were freeze-dried and re-extracted with 0.02 M sodium chloride solution. After centrifugation the supernatant was a yellowish-green fluorescent solution which was biuret-negative. Moreover, the nitrogen content of the insoluble proteins was increased.

A value of 0.33 percent phosphorus was obtained for the unfractionated proteins by extrapolating from the soluble and insoluble phosphorus contents. This value is in agreement with the data of Palmer and Weise (1933) who reported from 0.27 to 0.37 percent phosphorus. Separation of the membrane proteins concentrated the phosphorus in the soluble portion - 0.46 percent as compared to 0.23 percent in the insoluble fraction. The high phosphorus value for the soluble fraction could not be correlated with nucleic acid phosphorus.

The data in Table 6 show that the insoluble fraction contained 7.06 percent ash as compared to 2.08 percent in the insoluble portion. This is equivalent to 4.3 percent ash for the whole membrane protein. This value is high when compared to 3.22 percent ash obtained by Hare et al. (1952). Spectrographic evidence showed that the soluble fraction contained considerably more phosphorus, magnesium, calcium, and copper than the insoluble proteins. The relatively low nitrogen content of the soluble portion can be accounted for on the basis of a high mineral content and on the high sugar content as indicated by a strong, positive Molisch test. The intensity of the Molisch test varied among preparations of the insoluble fractions. Generally, a negative or very slightly positive Molisch reaction was noted. The Molisch reaction was especially weak or absent when the insoluble fraction was prepared from lyophilized proteins. The soluble protein solution reduced Fehling's solution after mild acid hydrolysis, but the quantity of cuprous oxide produced was small. Nevertheless, this indicated the presence of potential ketone or aldehyde reducing groups. Several attempts were made to chromatogram the sugars associated with the soluble membrane proteins. For this purpose, the procedure of Partridge (1948) was adapted. Most of the runs were fraught with technical difficulties, the difficulties being intense heading and streaking on the chromatograms. In spite of these adversities, presumptive evidence was obtained that pentose sugars and galactose or glucose were present.

Both protein fractions were nitroprusside-negative previous to heating. Following momentary heating of the insoluble fraction to 75°C., a strong positive nitroprusside test was obtained. Apparently the insoluble protein contained sulfhydryl groups which were activated by heat. This reaction was not observed with the soluble fraction. Insoluble proteins treated for solubilization were sulfhydryl-negative. Presumably, the reducing ability of the sulfhydryl groups was lost due to some chemical or physical interaction of the detergent with the proteins.

Ultraviolet analyses. Characteristic absorption peaks in both fractions were most intense at 278 millimicrons. Absorption in this region is characteristic for proteins containing aromatic groups. Morton (1954) reported the fat-globule membrane contained about 11 percent nucleic acids. In his method, acid-soluble phosphates were removed from the lipid free material by extracting with 0.5 N perchloric acid at 0°C. for four hours. The phosphorus which remained was assumed to be associated with nucleic acids and was extracted with 1 N perchloric acid at 80°C. Finally, he assumed that all absorption at 262 millimicrons was due to ribonucleic acid. Zittle et al. (1956b) were unable to find more than 2.4 percent nucleic acid. Neither Morton (1954) nor Zittle et al. (1956b) have isolated and demonstrated the presence of purine or pyrimidine groups associated with the nucleic acids. Thus, the presence of ribonucleic acid in the membrane appears to be questionable. If nucleic acids were present in appreciable quantities, intense absorption peaks would be found

in the 260 millimicron region. Spectrograms in Figures III and IV do not show characteristic absorption in this region. The soluble and insoluble fractions from this study were examined by Zittle (1956) who was unable to demonstrate the presence of nucleic acids.

The fact that nucleic acids might not be associated with the membrane seriously poses Morton's (1953, 1954) microsome theory. According to Novikoff, Podber, Ryan and Noe (1953) and Haurowitz (1955), microsomes definitely contain ribonucleic acids. More data are needed before concluding that the fat-globule membrane proteins contain nucleic acids. Evidence obtained in this study appears to preclude the presence of nucleic acids as a part of the membrane proteins.

Infrared analyses. The principle reason for obtaining an infrared analyses was to determine whether any differences were manifested between the two fractions in the absorption spectra. The only difference found was that the soluble portion showed a slight shoulder at 5.75 microns. Absorptions at 3.45, 6.85, and 7.27 microns were attributed to functional groups of the mineral oil in the Nujol mull. The peaks at 3.05 and 6.05 microns were due to amino stretch and bend, respectively. The absorption area at 9.50 microns was provisionally assigned to aryl phosphate linkages. Bellamy's (1954) text was used to identify the absorption areas.

Amino acids. Adjustment of the amino acid values to a whole membrane protein basis and subsequent comparisons with literature data authenticated the experimental values. This is illustrated in Table 7. Brunner et al. (1953a) and Hare

et al. (1952) reported 1.5 percent cystine for the membrane proteins, whereas a value of 2.43 percent was found in this study. This difference might be explained on the basis of different hydrolysis procedures. The method used in this study was a special procedure for cystine, adapted from Horn and Blum (1956), in which the proteins were hydrolyzed in an autoclave at 115°C. for only 30 minutes with 20 percent hydrochloric acid.

Both the soluble and insoluble fractions had amino acid compositions quite different from other reported milk proteins. The low glutamic acid and high arginine content of the insoluble proteins is unlike that of any other milk protein. Block and Vickery (1931) reported that proteins can be classed as keratins which are insoluble in dilute acids, alkalies, water, and organic solvents, but when hydrolyzed with acid yield a 1:4:12 ratio of histidine, lysine, and arginine. Block and Bolling (1939) classified insoluble protein which possessed a 1:1 ratio of lysine to arginine as pseudokeratins. For the insoluble protein preparation, a 1:1 ratio of lysine to arginine was found (Table 7). Thus, on the basis of insolubility, reaction to specific chemical reagents and amino acid composition, the insoluble fraction can be provisionally classified as a pseudokeratin. Certainly one can postulate that a certain amount of protein, resistant to inherent proteolytic enzymes and insoluble in the milk serum, might surround and stabilize the fat globules.

The amino acid composition of the soluble fraction was unlike any of the known milk proteins. This fraction contained

more cystine and less glutamic acid than any of the reported milk proteins. Interestingly, the soluble fraction contained quantitatively less amino acids than the insoluble fraction. However, the soluble fraction contained considerable non-protein materials which might have contributed to the destruction of amino acids upon hydrolysis. Actually, the amino acid composition of the two fractions was not radically different. The greatest difference between the fractions was found in the arginine, methionine, and valine contents.

Quantitative determination of amino acids by paper chromatography is relatively new, but the accuracy of this method has been substantiated by Block and Weiss (1955). The importance of using good analytical reagents and saturating the chromatographic chamber before the run was made in this study. The values obtained for phenylalanine and proline were questionable and are not reported, but the method appears to be adequate for these determinations. With the exception of proline, the color of the developed chromatograms was quite stable when stored in the dark. Proline produced a green, unstable color when developed with isatin which could not be eluted with the extractant used. Apparently a densitometer should be used in conjunction with proline determinations.

Although the amino acid composition of the soluble fraction did not permit classification of this protein, there are data which indicate that it is globulin in nature. On the basis of sedimentation velocity studies (S_{20} = 7.3),

Brunner et al. (1953b) provisionally classified the fat-membrane protein as globulin-like. Some exploratory studies (unreported) of the soluble proteins with a Spinco Model E Ultracentrifuge substantiated the above data; however, a low molecular weight component of low concentration was also observed. Thus, on the basis of sedimentation studies, insolubility in half-saturated ammonium sulfate, and an isoelectric zone near pH 5.0, the term globulin-like best fitted the soluble fraction.

The primary purpose of determining amino acid composition of the two fractions was to compare both fractions and the individual fraction compositions with other known proteins. Since the nitrogen values of each fraction indicated the presence of non-protein materials, minimum molecular weights were not calculated.

Spectrographic analyses. The residue from the ash determinations was taken-up in 1:1 hydrochloric acid and submitted for spectrographic analysis in the carbon arc of a Hilger spectrograph. A Jarrell-Ash microphotometer was used to evaluate the spectrograms in which cobalt was the internal standard. Qualitative and approximate quantitative data for identified minerals are given in Table 9. All of the elements reported here have been demonstrated spectographically in whole milk (Dingle and Sheldon, 1938). These researchers also reported rubidium, lithium, barium and strontium in whole milk. Under the conditions of this study, these four elements could not be demonstrated in the membrane proteins. Gehrke,

Baker, Affsprung and Pickett (1954) reported semi-quantitative spectrographic data for the trace elements in whole milk. In the instances where comparisons were possible, the fat-globule membrane proteins possessed higher concentrations of specific elements than those reported for whole milk. This indicates that many of the trace elements in whole milk are concentrated in the membrane.

Calcium was found in the fat-membrane material by Hattori (1925), but Palmer and Weise (1933) could not confirm this finding. These workers believed that all of the calcium present was dialyzable. Calcium was detected spectrographically in this study in proteins which had been exhaustively dialyzed against distilled water. Moreover, if the calcium were a contaminant, one would expect to find equal quantities in each fraction. As with most elements associated with proteins, the role of calcium in the membrane proteins is obscure. On the basis of known biological activity associated with the membrane, the identification of iron, magnesium and molybdenum was not surprising. Richert and Westerfeld (1953) isolated xanthine oxidase from cream which contained 0.03 percent molybdenum. It is interesting to note that xanthine oxidase and molybdenum were both concentrated in the insoluble proteins. Richert and Westerfeld (1953) believed that molybdenum was a part of the xanthine oxidase molecule. Morton (1954) reported that cytochrome-c was associated with the membrane proteins; thus, the presence of iron was suspected. Probably the concentration of magnesium in the soluble fraction

was related to its high phosphatase activity.

A limited amount of information is in the literature showing a complete elemental composition of milk protein residue ash. The data in Table 9 represent the first spectrographic analysis of the membrane proteins and indicate a wide difference in the mineral distribution between the two fractions. Although the value of these data is of limited use, their potential significance can not be predicted.

SUMMARY AND CONCLUSIONS

Essentially, this research was divided into two sections: first, to isolate the fat-globule membrane proteins with a minimum of undesirable changes, and second, to study the physical, chemical, and biological characteristics of the isolated proteins. Five concentrations of cold ethanol, four of cold n-butanol, and room temperature n-butanol were investigated as agents to disrupt lipoprotein complexes. Proteins pretreated with 35 percent ethanol (final concentration about 26 percent) were most amenable to physicochemical studies. Under the conditions of this study, ethanol pretreatment was more satisfactory than either cold or room temperature n-butanol for disrupting the membrane lipoprotein complex.

The membrane proteins were separated into soluble and insoluble fractions based upon solubility in a 0.02 M sodium chloride solution. These fractions differed widely on the basis of physical, chemical and biological properties. The soluble fraction had a strong Molisch reaction, reduced Fehling's solution subsequent to mild acid hydrolysis, and was sulfhydryl-negative after heating to 75°C. Nitrogen values varied with individual preparations from 9.5 to 11.5 percent. An average of seven-fold more phosphatase activity was found in the soluble than in the insoluble fraction. A single skewed component was observed in alkaline buffers, whereas three closely associated components were evident in

acidic buffers when the soluble fraction was studied electrophoretically. Regression equations $Y = 8.840 - 1.762X$, $Y = 7.250 - 1.455X$, and $Y = 4.663 - 0.900X$ were calculated which gave isoelectric points at pH 5.02, 4.98, and 5.18, respectively.

On the basis of sedimentation velocity data, insolubility in half-saturated ammonium sulfate and an isoelectric zone near pH 5.0, the soluble proteins were tentatively classified as globulin in nature.

The residual fraction was insoluble in dilute acids and bases, 25 percent sulfuric acid and 6 and 8 M urea. Strong reducing agents commonly used to attack disulfide-linked proteins and certain detergents were found as capable solubilizing agents. Sodium sulfide and sodium lauryl sulfate were found to be good solubilizing agents.

The nitrogen values ranged from 12.9 to 13.9 percent for the insoluble protein. A qualitative sulfhydryl-positive reaction was obtained upon heating the protein moiety to 75°C. In contrast to the white color of the soluble fraction, the insoluble material was a reddish-brown. The insoluble fraction contained 5.6 times more iron, 25 times more molybdenum, and 10 times more xanthine oxidase activity than the soluble fraction.

Electrophoretic analyses of the insoluble fraction were carried out on solubilized-protein in alkaline buffers. Sodium sulfide-solubilized material showed one homogeneous component and the detergent-solubilized protein had two

prominent and one minor components.

On the basis of insolubility in the usual protein solvents, reactivity to specific reducing agents, and amino acid composition, the insoluble proteins were provisionally classified as pseudokeratin in nature.

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