ELECTROPHORETIC AND PARTIAL CHEMICAL CHARACTERIZATION OF BOVINE MILK FAT GLOBULE MEMBRANE PROTEINS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
MICHAEL E. MANGINO
1973

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ABSTRACT

ELECTROPHORETIC AND PARTIAL CHEMICAL CHARACTERIZATION OF BOVINE MILK FAT GLOBULE MEMBRANE PROTEINS

By

Michael E. Mangino

The lipid fractions of milk fat globule membrane (MFGM) have been characterized by a number of researchers, while the protein fractions have received little attention as to their physical characteristics. This is due, in part, to the resistance of these protein fractions to solublization by buffers usually employed in protein chemistry.

MFGM material was sequentially extracted with 0.6 M KCl and centrifuged to yield five 100 S supernatant and pellet fractions. These fractions were characterized both chemically and physically by the use of polyacrylamide gel electrophoresis in a SDS system.

The original extraction and centrifugations resulted in a nearly equal distribution of lipid and protein material between the supernatant and pellet fractions. Subsequent extractions of the pellet fractions resulted in a much greater loss of lipid than protein material to the supernatant fractions. The protein to lipid ratio increased from 0.83 for fraction 1-P to 1.65 for fraction 5-P while the phospholipid to protein ratio decreased from 0.31 for fraction 1-P to 0.24 for fraction 5-P.

Concomitant with these compositional changes the pellet fractions decreased in solubility with each extraction. Examination of the electrophoretograms obtained for these fractions indicated that the decreasing solubility was due to the aggregation of low molecular weight

polypeptides into larger, less soluble ones.

It was concluded that the removal of lipoidal material from the pellet fractions caused an irreversible aggregation of the protein moieties and that this aggregation was the main cause for the decreased solubilities of these fractions.

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Michael E. Mangino

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1973

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. J. R. Brunner, Professor of Food Science and Human Nutrition, for his patience, counsel and inspiration during the course of this study.

Appreciation is also extended to Dr. C. M. Stine and Dr. H. A. Lillevik for aid in preparation of the manuscript.

Acknowledgment is due to Ursula for aid and counsel on numerous technical matters.

The author also expresses his gratitude to his wife, Lynn, for her aid and encouragement during the course of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The fat globules that occur in milk are secreted from the mammary gland by a method described as reverse pinocytosis. At the time of secretion, these fat droplets are surrounded by a membrane layer about 90A thick.

Recently, workers employing the electron microscope, comparative chemical analysis and enzymatic studies have presented strong evidence in support of the theory that the milk fat globule membrane arises from the plasma membrane of the mammary gland. Studies of the milk fat globule membrane should give insight into the composition and structural organization of other, more difficult to isolate, membrane systems.

The composition of the lipid moieties of the milk fat globule membrane has been well elucidated while the protein components have long resisted characterization, partially due to their inherent insolubility.

Several workers have fractionated membrane proteins on the basis of solubility in different solvent systems. Other than chemical analysis, little data are available to explain these solubility differences.

It was the purpose of this study to separate the milk fat globule membrane proteins on the basis of solubility and to characterize the resulting fractions chemically and electrophoretically.

REVIEW OF THE LITERATURE

Historical Survey of the Fat Globule Membrane

Ascherson (1840) was the first to report the existence of a thin membrane surrounding the fat globules in milk which he labeled the "haptogen membrane." He believed that it arose from the condensation of albumin at the fat/plasma interface.

Babcock (1885) examined fat globules before and after churning and could detect no change in their appearance, nor could he detect fragments in the serum. He therefore concluded that no membrane existed. Later he (Babcock, 1889) reported the existence of a protein that he felt was responsible for the clustering of fat globules during the creaming process, which could also serve as a membrane. He likened the creaming of milk to the coagulation of blood and called this protein lacto-fibrin.

Storch (1897) realized that it would be possible for many milk proteins to adhere to the fat globules and devised a method of washing the fat globules. Ether extraction of these washed globules yielded a residual mucin-like protein which gave a positive test for carbohydrate.

Hattori (1925) found that the "hapteins" were different than other milk proteins on the basis of their physical and chemical characteristics. He described these proteins as keratin-like and noted their extreme lack of solubility.

Titus (1928) and co-workers isolated fat globules without any previous washing. Examination of the isolated protein led them to

conclude that the main protein of the fat globule membrane was casein.

Palmer (1924, 1933, 1936) and his co-workers in a series of experiments were the first to demonstrate that the membrane protein was complexed with phospholipid. The result of these experiments was the theory that the membrane was composed of a single globulin-like protein linked to various amounts of phospholipid.

Brunner (1953) and co-workers showed that the protein material of the membrane was altered during homogenization. The unhomogenized membrane protein exhibited sedimentation characteristics similar to lactoglobulins in the analytical ultracentrifuge. On the basis of this observation, the protein was provisionally classified as globulin-like in nature.

Herald and Brunner (1957) fractionated the membrane into two components by centrifugation at 25,000 x g in 0.02 M sodium chloride for one hour. The pellet obtained was insoluble in phosphate and veronal buffers and was termed "insoluble." The mucoidal supernatant was designated as the "soluble" fraction. This represented the first demonstration that the membrane complex contained more than one protein entity. The insoluble fraction was tentatively classified as a pseudokeratin.

For a more comprehensive review of the membrane proteins including accepted methods of preparation, the enzymatic content and the possible origin and structure of the fat globule membrane, the reader is referred to the review of Brunner (1973).

Isolation and Characterization Of the Insoluble Membrane Fraction

After their classification of the protein that was not soluble in 0.02 M sodium chloride as the insoluble fraction, Brunner and Herald (1957) reported the heterogeneous nature of this fraction. Solubliza-tion of this material by various dissociating agents and subsequent analysis by moving-boundry electrophoresis showed it to be made of more than one species.

Thompson and Brunner (1959) substantiated the earlier observation of Storch that the membrane proteins were glycoproteins. Thompson (1960) further examined the insoluble membrane protein and on the basis of results obtained from low angle x-ray diffraction described this protein as fibrous in nature.

Alexander and Lusena (1961) fractionated membrane material into five pellets and a soluble fraction by treating washed cream that was previously stored at -20 C with 2% deoxycholate and employing differential centrifugation on the resulting mixture. They found that the lipid to protein and phospholipid to total lipid ratios were much higher in the supernatant fraction than in any of the pellets. Physical character-ization of the pelleted material was not attempted because of its insolubility in normal dispersing agents.

Harwalker and Brunner (1965) studied the effects of dissociating agents on the membrane fractions and concluded that the insoluble fraction was a heterogeneous mixture of lipoproteins. Analytical

ultracentrifugal examination of the solublized material showed a large number of boundries indicating heterogenity. They concluded from the effects of these bond-specific dissociating agents on the insoluble membrane fraction that hydrophobic bonding was the principal stabilizing factor in the membrane complex with significant contributions from covalent disulfide bonds.

In an experiment similar to that of Alexander and Lusena, Hayashi and Smith (1965) treated washed cream with 1% deoxycholate. After centrifugation, 45% of the membrane protein was in the supernatant along with 67% of the total membrane phospholipid. The pellet protein fractions contained a much higher protein to phospholipid ratio. These workers hypothesized that the membrane was composed of an insoluble protein region covered by a more soluble outer layer.

In a subsequent experiment, Hayashi (1965) and co-workers showed the deoxycholate solublized proteins to be heterogeneous by separating them into three density classes. No attempts were made to classify the insoluble protein fractions.

Chien and Richardson (1967) used a combination of physical stirring and centrifugation to separate the membrane proteins into five fractions. The easily removed material accounted for approximately half of the original protein and they concluded that this came from an easily soluble outer layer. They thought that the remainder of the protein came from an insoluble inner layer of the membrane.

Swope and Brunner (1969) used differential sedimentation to separate the undissociated membrane proteins into a soluble supernatant containing approximately 6% of the total protein and three pellet fractions. They found that as the density of these pellets increased,

the protein to lipid ratio increased and the carbohydrate to protein ratio decreased.

It should be noted that all the methods thus far described for the characterization of membrane proteins have yielded data pertaining to the chemical composition of the various fractions obtained.

Attempts to physically characterize the membrane system have met with very limited success and only indicated that the membrane is composed of a heterogeneous mixture of glyco-lipoproteins. The lack of data pertaining to the physical composition of the membrane proteins can be attributed to their resistance to solublization.

Gel Electrophoresis of Membrane Proteins

Most membrane proteins have long resisted characterization by gel electrophoresis because of their inherent insolubility. Harwalker and Brunner (1965) found that the bulk of fat globule membrane protein applied to gels did not enter the running gel even after treatment with various dissociating agents. This prompted Brunner (1969) to conclude that gel electrophoresis, under normal conditions, was not a satisfactory technique for studying the membrane complex.

Takayama, et al. (1966) developed a system containing phenol, acetic acid, water (2:1:1, w/v/v), 2 M urea that solublized the proteins of the mitochondrial transport system. The solublized proteins were electrophoretically separated on acid gels (7.5% acrylamide, 35% acetic acid and 5 M urea) with reproducible patterns.

This method was modified by Ray and Marinette (1971) by the addition of 5% mercaptoethanol to the solublizing solution. The acrylamide content of the gels were lowered to 6% and the urea to 4 M.

This modification when used on rat liver plasma membranes gave more and sharper zones than did the original system.

Keenan, et al., (1970) employed the original system of Takayama et al., (1966) to compare the proteins of the fat globule membrane to those of the mammary gland plasma membrane. They found that the two membrane systems were almost identical in protein distribution and that the major component of both systems was the same.

The introduction of a method to estimate the molecular weights of proteins and their subunits on polyacrylamide gels by Shapiro, et al., (1967) added a new dimension to disc gel electrophoresis. These authors reported that the binding of sodium dodecyl sulfate (SDS) to proteins negates their native charge and causes them to separate on the basis of their molecular weights only. They compared the relative mobility (i.e., the ratio of the mobility of the protein to that of the tracking dye) of ten proteins of known molecular weights and achieved an essentially linear relationship. This system incorporated 1% mercaptoethanol into the sample buffer to break disulfide bonds and insure that the protein standards were in monomeric form. The combination of SDS and mercaptoethanol proved to be a very powerful solublizing agent.

Weber and Osborn (1969) further investigated this system by comparing the relative mobilities of 40 proteins of known molecular weights. They found that by altering the gel concentration or the degree of cross-linkage within the gel that molecular weights within the range of 11,000 to 220,000 could be obtained with an accuracy of $\pm$ 10%.

Weber and Kuter (1971) explained this phenomenon by showing that all proteins examined bound 1.4 g of SDS per gram of protein regardless of the size or shape of the protein molecule. The binding of a specific

amount of SDS per weight of protein gave all proteins the same net charge and converted them into rod shaped molecules whose length was proportional to their molecular weights.

Rodbard and Chramback (1971) in a theoretical treatment of molecular size estimations by use of polyacrylamide gels noted points of caution that should be taken. They point out that if the assumptions of Weber and Kuter are true, that there is no mathematical basis for being able to determine the molecular weight of a molecule by electrophoresis under any conditions if only one gel concentration is used. They cautioned that when comparisons are made between different systems, the retardation coefficient of the molecules should be compared and not some derived function of it, viz., its molecular weight. They further stated that claims of accuracy in the SDS gel system of $\pm$ 10% may not be valid because rigorous statistical treatment of the rather limited data has not been performed. Their final point was that at least two gel concentrations should be used if molecular weight estimates are to be made.

Heriman and Phillips (1970) applied the system of Shapiro to the membranes of rough and smooth endoplasmic reticulum isolated from rat liver. They found the system gave reproducible results as long as precautions were taken to insure that the membranes were always prepared in the same way. This led to the conclusion that meaningful comparisons of the protein profiles of a given system are valid only as long as the methods used to isolate the proteins are identical.

A lipoprotein fraction obtained from erythrocytes was studied by Juliano and Rothstein (1971). They found this previously insoluble fraction to be totally soluble in a solution containing SDS and mercaptoethanol. They reported that the solution used to solublize this fraction

completely dissociated the lipid-protein complex.

The outer membrane of Escherichia coli was solublized with SDS into lipid and protein components by Bragg and Hou (1972). Removal of the SDS by dialysis caused the reassociation of these components into membrane-like structures. When these solublized membranes were electrophoresed according to the method of Weber and Osborn, a reproducible pattern was obtained. When the pH of the cathodic buffer was changed by the addition of NaOH from pH 7.2 to pH 11.0,a reproducible pattern was also obtained. The patterns obtained between the two systems, however, were not identical. Thus, another variable must be considered when the protein patterns of the same substance are compared and the electrophoresis is performed under slightly different conditions.

Allen and Crumpton (1972) used 5% SDS to solublize the membranes of human thymocytes. They stained gels both with coomassie blue and with periodate-Schiff reagent, obtaining 20 coomassie blue positive zones and six that were positive for carbohydrate. The carbohydrate-positive bands corresponded to six of the protein bands, indicating that these were glycoproteins. A carbohydrate-positive, protein-negative zone was found near the ion front of each gel. Butanol extraction of the membrane removed 98% of the lipid and 62% of the neutral sugar. When the butanol extracted protein was electrophoresed, the fast migrating band was no longer present, and was, therefore, considered to be a glycolipid.

In studies on the outer mitochondrial membrane of beef heart, Hayashi and Roderick (1972) used the method of Shapiro. Carbohydrate staining indicated only one positive zone migrating ahead of all the protein zones and was considered to be a glycolipid. These authors concluded that this portion of the membrane, while being reported to

contain carbohydrate, actually contained no glycoproteins.

Keenan and Huang (1972) employed a pH 7.0 phosphate buffer containing 1% mercaptoethanol and 1% SDS to solublize the fat globule membrane proteins. This mixture was incubated with stirring at 37 C for four hours. The remaining insoluble material was removed by centrifugation and the samples applied to the gel system of Weber and Osborn (1969). They reported that the fat globule membrane complex contained 14 polypeptide units ranging in molecular weights of from 11,500 to 102,000 daltons. The main zone had a molecular weight of 68,000 daltons.

Koblyka and Carraway (1972) used almost identical conditions to separate the polypeptides of the fat globule membrane. The major differences between these two reports as to methods employed were:

1. Keenan and Huang used freshly drawn warm milk; Koblyka and Carraway used chilled milk.
2. Keenan's group used a 10% acrylamide gel, whereas Koblyka and Carraway used a gel concentration of 5%.
3. Koblyka and Carraway assumed that all polypeptides that showed molecular weights of less than 50,000 daltons were contaminants from the milk serum, while Keenan and Huang assumed that their preparation was free of contaminants and all bands should be considered part of the membrane complex.
4. Koblyka and Carraway used both a protein and a carbohydrate stain, while Keenan and Huang used only a protein stain.

Koblyka and Carraway reported the presence of six coomassie blue positive zones ranging in molecular weights from 53,000 to 240,000 daltons. They also reported the presence of six carbohydrate containing

polypeptides, three of which were definitely different from the coomassie blue positive zones. The main component in their system had a molecular weight of 66,000 daltons.

These two reports are significant because they contain the first published data concerning the actual number of polypeptides in the fat globule membrane complex and give molecular weight estimates for these proteins. The disparity in the results, however, indicates the need for further investigation of the membrane system.

EXPERIMENTAL

Chemical Analysis

Nitrogen

Duplicate nitrogen analyses were performed in a semi micro-Kjeldahl apparatus. Ten to twenty mg of dried sample was mixed with 4 ml of digestion mixture consisting of 5.0 g $CuSO_4.5H_2O$ and 5.0 g SeO in 500 ml of concentrated H_2SO_4. Digestion was carried on for one hour or until the solution was clear. The contents of the digestion flasks were allowed to cool for 30 min and 1 ml of 30% H_2O_2 was added. Digestion was continued for another hour and allowed to cool for 30 min. The flasks were then rinsed with 10 ml of deionized water and allowed to cool for an additional 30 min. The mixture was neutralized with 25 ml of a 40% NaOH solution and the released ammonia was steam distilled into 15 ml of a 4% boric acid solution containing 5 drops of indicator solution. The indicator solution was made by dissolving 400 mg of bromocresol green and 40 mg of methyl red in 100 ml of 95% ethanol. The distillation was continued until a final volume of 75 ml was reached. The ammonia-borate complex was titrated with 0.020 N HCl previously standardized with tris hydroxymethyl aminomethane. A reagent blank was determined and subtracted from the sample values. The average recovery of tryptophan was 97.2%.

Hexose

The phenol-sulfuric acid method of Dubois, et al. (1965) was used to determine hexose. Five hundred to one thousand µg of sample in 1 ml

of water was mixed with 1 ml of 5% mixture of redistilled phenol and water in a 25 ml Erlenmeyer flask. Five ml of concentrated H_2SO_4 was added rapidly while the flask was agitated to insure maximum mixing and heat development. The samples were allowed to stand at room temperature for 10 min and were then kept in a 25 C water bath for an additional 20 min. Transmittance was read at 490nm. A standard curve was constructed to cover a range of 0 to 50 µg of a galactose-mannose (1:1) mixture. A reagent blank was prepared by omitting the protein from the reaction mixture.

Hexosamine

The method of Johnson et al. (1960) was used to determine the hexosamine content of the samples. One to two mg samples were weighed directly into 5 ml ampoules. One ml of 4 N HCl was added and the samples were frozen in a mixture of dry ice and ethanol. The ampoules were evaporated, refrozen and sealed under a vacuum. Standard mixtures of glucosamine-galactosamine (1:1) were treated in the same manner as the samples to serve as recovery standards. The samples were placed in a hot air oven at 100 C and hydrolyzed for six hours to release hexosamine. The hydrolyzed samples were transferred to a micro-Kjeldahl flask and the ampoules were rinsed with 1 ml of 4 N NaOH and two 1 ml rinses of deionized water.

Acetylacetone reagent was prepared by adding 1 ml of distilled acetylacetone to 25 ml of 1 M Na_2CO_3 solution and 20 ml of H_2O. The pH was adjusted to 9.8 and the solution made up to 50 ml with deionized water. The acetylacetone reagent must be used within 30 min of its preparation. Ehrlich's reagent was prepared by dissolving 2 g of p-dimethylaminobenzaldehyde into 250 ml of a 3.5% solution of HCl in ethanol.

Five and five-tenths ml of the acetylacetone solution was added to each digestion mixture. The final pH of this mixture should be between 9.5 and 10.0. The flasks were stoppered and placed in a boiling water bath for 20 min. After cooling, the flasks were attached to a micro-distillation apparatus and the chromogen was distilled into a 10 ml volumetric flask containing 7.5 ml of Ehrlich's reagent. Transmittance was read after 30 min at 548 nm.

A standard curve containing 0.0 to 20 µg was constructed from a mixture of glucosamine-galactosamine (1:1). The average recovery was 97% of the standard mixture. A reagent blank contained 1 ml of 4 N NaOH, 1 ml of 4 N HCl and 2 ml of H_2O that was treated in the same manner as the samples.

Sialic Acid

Warren's (1959) thiobarbituric acid method as modified by Marier, et al. (1963) was used to determine the sialic acid content of the proteins. Sialic acid was released from the samples by hydrolyzing 5 mg samples in 5 ml of 0.1 N H_2SO_4 for 40 min at 80 C. Aliquots of from 0.1 to 0.5 ml were made to a total volume of 0.5 ml with deionized water in a conically-shaped test tube. One-tenth ml of a sodium metaperiodate solution (0.2 M in 9M H_3PO_4) was added. The samples were mixed and allowed to stand at room temperature for 20 min. Because of the small volumes employed, adequate mixing is very important. One ml of a sodium arsenite solution (10% in 0.5 M Na_2SO_4 and 0.1 N H_2SO_4) was added and the tubes were shaken until the yellow brown-color disappeared. Three ml of a barbituric acid solution (0.6% recrystallized barbituric acid in 0.5 M Na_2SO_4) was added. The tubes were agitated vigorously to extract the chromophore into the cyclohexanone layer. The emulsion

was broken by centrifugation and transmittance of the upper layer was monitored at 549 nm. A standard curve was prepared using N-acetyl neuraminic acid as a standard in concentration of from 0 to 20 µg. Deionized water was used instead of sample in determinations serving as reagent blanks.

Total Lipid

Total lipid was determined by a micro-modification of the method of Monjonnier and Troy (1925). Duplicate samples of from 10 to 25 mg were weighed directly into conically-shaped centrifuge tubes. One and five-tenths ml of a 2% KCl solution was added to each tube along with 2 drops of concentrated NH_4OH. The tubes were capped and allowed to stand at room temperature for 2 hr to solublized the samples. One and five-tenths ml of 95% ethanol was added to each tube and the tubes were capped and agitated for 1 min. Two and five-tenths ml of ethyl ether was added to each tube; the tubes were capped and agitated for 30 sec. The pressure was released and agitation was continued for an additional 30 sec. Two and five-tenths ml of petroleum ether was added to each tube followed by the agitation procedure described above.

The tubes were centrifuged to separate the phases and the upper solvent phase was removed and added to a previously tared aluminum dish that was held at 45 C on an electric hot plate. Each tube was rinsed with 1 ml of a mixture of ethyl ether and petroleum ether (1:1) and the extraction procedure was repeated twice. The resulting solvent layers were added to the dish and evaporated to dryness. The dishes were then placed in a vacuum oven at 100 C. After 30 min, they were transferred to a dessicator, allowed to cool, and weighed. The solvent blanks were

treated in the same manner and their average weight was subtracted from the weight of each sample. Results were expressed in percentages of total sample weight.

Phospholipid

The limited amount of lipid material recovered by the lipid extraction procedure negated the usually employed lipid-phosphorus procedure. Thus, it was assumed that all phosphorous measured in a given membrane fraction was a constituent of the phospholipid moiety. Swope and Brunner (1968) demonstrated that the amount of phosphorous found in the protein portion of three membrane fractions was fairly low and nearly constant between fractions. They believed that this phosphorous was attributable to residual phospholipid material.

A modification of the method of Ames (1966) was used to determine the phosphorous content. The method as described is capable of determining 0.01 μM of phosphate.

Duplicate aqueous samples, containing 200 to 250 μg of membrane and 0.06 ml of a 10% solution of $Mg(NO_3)_2 \cdot 6H_2O$ in 95% ethanol, were heated over a Bunsen burner in a Pyrex test tube until the brown fumes disappeared. Six-tenths ml of 0.5 N HCl in deionized water was added and the tightly stoppered tubes were heated in a boiling water bath for 15 min and allowed to cool.

To each tube was added 1.4 ml of a freshly prepared solution containing 1 part of a 10% ascorbic acid solution and 6 parts of a 0.42% ammonium molybdate solution in 1 N H_2SO_4. The tubes were incubated at 45 C for 20 min and the contents volumetrically diluted to 5.0 ml with deionized water. Transmittance was monitored at 660 nm.

Standard solutions containing from 0 to 3.72 μg phosphorus along with a reagent blank were treated in a manner identical to that of the samples to construct a standard curve. The amount (μg) of phosphorus in the samples was multiplied by 25 to give the phospholipid equivalents in μg.

Lowry Protein

The method of Lowry, et al. (1951) was used for analysis of the protein content of non-lyophilized membrane fractions.

Five-tenths ml of each sample was placed in a test tube with 0.5 ml of 0.2 M NaOH (final concentration of 0.1 M NaOH in 1 ml) and was allowed to stand at room temperature for 30 min.

Five ml of alkaline copper solution (made freshly by the dilution of 1 ml of a solution of 0.5% cupric sulfate and 1% sodium tartrate to 50 ml with a 2% sodium carbonate solution) was added to each sample and the mixture allowed to incubate at room temperature for 10 min.

After incubation, 0.5 ml of Folin reagent diluted 1:1 with deionized water was added with rapid agitation. Transmittance was read at 750 nm after 30 min. A standard curve was constructed with crystallized bovine serum albumin in the range of from 0 to 250 μg. The standard protein and a reagent blank were treated in the same manner as the membrane fractions.

Physical Methods

Preparation and Fractionation of Membrane Proteins

The milk used in this work was obtained from the Michigan State University dairy herd which consisted mainly of Holstein cows. The milk was collected warm, at the time of milking and separated within

one hour at 37 C. The separated cream was diluted with three volumes of distilled water kept at 37 C and reseparated. This procedure was repeated three times to insure adequate removal of skim milk proteins (Swope, 1968). The washed cream was stored at 4 C overnight and churned in Erlenmeyer flasks on a rotary shaker at room temperature. After the emulsion was broken, the aqueous portion was filtered through four layers of cheese cloth. The unchurned butter granules were removed by centrifugation at 1000 x g for 30 min. The resulting aqueous suspension was designated as the original membrane material (0).

Figure 1 represents the scheme used to separate the membrane material into five pellet (1-P through 5-P) and five supernatant (1-S through 5-S) fractions. In all cases the pellets were resuspended in a volume of 0.6 M KCl equal to the original volume of the 0 fraction and allowed to stir overnight at 4 C. Samples of the original membrane material, the supernatants and the pellets were dialyzed exhaustively against several changes of distilled water at 4 C. Portions of the dialyzed fractions were saved for physical analysis and the remainder lyophilized for subsequent chemical analysis.

The sedimentation values for the pellets were determined by a rearrangement of the equation of Trautman (1961):

$$S = \frac{10^{13} \ln (r_a/r_b)}{\omega^2 T} ;$$

to yield

$$ST = \frac{10^{13} \ln (r_a/r_b)}{\omega 2} ;$$

where S = Svedberg units

T = time in hours

r_a = the radius from the center of rotation (cm) to the bottom of the liquid

r_b = the radius from the center of rotation (cm) to the air-liquid meniscus, and

ω = the angular velocity in rad/min = 2π (rpm).

For the 30 rotor:

r_a = 10.5 cm

r_b = 5.0 cm

rpm = 30,000

would yield

$$ST = \frac{10^{13} \ln (10.5/5.0)}{(2\pi)^2 (30,000)^2} = 208.$$

The samples were centrifuged at 30,000 rpm for 2 hr which yields:

S(2) = 208

S = 104 S pellet.

Polyacrylamide Disc-Gel Electrophoresis

Disc electrophoresis was performed in the acid system of Takayama (1966) and the sodium dodecyl sulfate (SDS) system of Weber and Osborn (1969). Electrophoresis was conducted in a 12 sample Buckler apparatus. Gels were stained for protein for at least 2 hr in a solution of coomassie blue as described by Weber and Osborn (1969) and destained by diffusion in a Bio Red destainer. Gels were stained for carbohydrate by the method of Zacharias (1969).

Acid Gels

The acid system employed gels formed with 7.5% acrylamide, 35% acetic acid and contained 5 M urea. The samples were solublized in a system of phenolacetic acid-water (2:1:1, w/v/v) with the aid of ultrasonification. Samples were applied to the gels followed by a layer

of the solublizaing solution. The buffer tanks were filled with 10% acetic acid with the lower tank serving as the cathode. Electrophoresis was carried out at room temperature for 2.5 hr with a constant current of 5 mA/ tube. The gels were stained and destained as previously described.

10% SDS-Gels

The 10% acrylamide, sodium dodecyl sulfate (SDS) gels were run according to the method of Weber and Osborn (1969) with two exceptions:

1. The tank buffer was diluted 1:1 with deionized water,
2. The samples and standards were dissolved and applied to the gels in 0.05 M phosphate buffer (pH 7.1) containing 1% SDS and 1% mercaptoethanol.

The samples were layered over the gels by increasing their densities with sucrose. Electrophoresis was carried out at room temperature with a constant current of 8 mA/ tube for 4 hr. The lower electrode served as the anode. Gels were stained for both protein and carbohydrate as previously described.

5% SDS-Gels

In order to obtain more reliable estimates of the molecular weights of large polypeptides, 5% acrylamide gels were used. For these gels commercial Cyanogum 41 was used in place of acrylamide and bisacrylamide. The samples were dissolved and electrophoresed in exactly the same manner as described above for the 10% SDS-gels. The time of electrophoresis was reduced from 4 to 3 hr.

Molecular weights of protein subunits were estimated from a plot of the relative mobility versus the log of the molecular weights of

protein standards run on both concentrations of SDS gels. The standards used were β-lactoglobulin (18,400), ovalbumin (43,000), catalase (60,000), and phosphatase A (94,000). Relative mobilities were calculated by measurement of the protein zone migration distance, dye migration distance and the length of the gels from the following relationships:

$$\text{Relative mobility} = \frac{\text{distance protein migrated}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before destaining}}{\text{distance dye migrated}}.$$

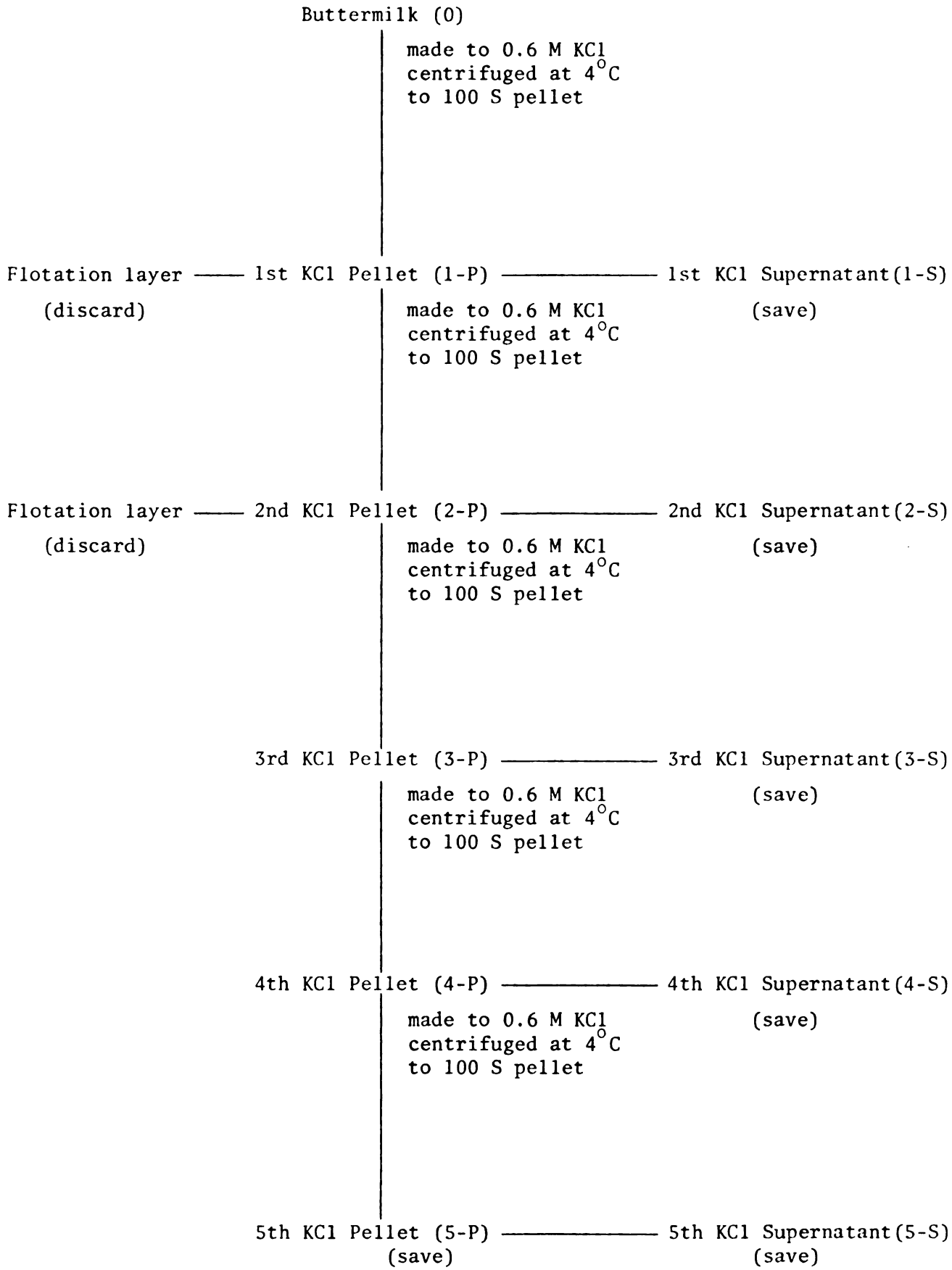


Figure I. Diagram for the sequential extraction of membrane material with 0.6 M KCl

RESULTS

Distribution of Protein, Lipid, and Phospholipids Between Membrane Fractions

The recoveries of protein, lipid, and phospholipid for each membrane fraction are presented in Table I. The first column gives the actual protein recovery as the percentage of the original material. There was a 7.7% loss of the toal protein after the first centrifugation, probably due to the discarding of the flotation layer. To better represent the manner in which the recovered protein was distributed between the various fractions, the data in the second column represent the protein recovery for each fraction when the sum of the protein contents of 1-S and 1-P are taken as 100%. The third and fourth columns of Table I show the distribution of lipid and phospholipid between the membrane fractions. No corrections were made for losses or gains of these components. The recovery of material in the supernatant fractions 3-S through 5-S was too low to allow for accurate lipid or phospholipid determinations.

Chemical Composition Of Membrane Fractions

Table II represents the percentage composition of the membrane fractions in terms of protein, lipid, hexose, sialic acid, and protein + total carbohydrate.

The average nitrogen values of membrane protein fractions reported by Swope and Brunner (1970) ranged from 14.01% to 14.14% as compared to

approximately 16.0% for normal milk proteins. The difference presumably being due to the fact that membrane proteins are actually glycoproteins (Thompson and Brunner, 1959). For purposes of calculating the total protein content of the membrane fractions, it was assumed that all the carbohydrate detected was conjugated to the proteins. In all subsequent comparisons of components to protein, the protein content will be taken as the sum of the protein and carbohydrate moieties. Calculations derived from the data of Table II show, in fact, that if the nitrogen contents of fractions 1-S and 1-P are divided by the protein + carbohydrate content of these fractions, the resulting percentages of nitrogen are 14.5% and 14.2% respectively. These values are in agreement with the aforementioned data of Swope and Brunner and strengthens the assumption that the total protein content of the membrane fractions is best represented by the sum of the values for protein and carbohydrate.

Table III expresses the same data of Table II as component ratios rather than percent composition for the chemical components of the membrane fractions.

Molecular Weights Determined By Gel Electrophoresis

The molecular weights obtained for the polypeptides of the membrane fractions are tabulated in Tables IV, V, VI, and VII. Tables IV and V give the values for the pellet fractions in 10% and 5% gels respectively. Tables VI and VII give the values for the supernatant fractions in the same gels.

Gel Electrophoretic Patterns

Acid System

The electrophoretic patterns obtained for fractions 0, 1-S, 2-S,

1-P and 5-P, where the samples were solublized with phenol- acetic acid-water, (2:1:1, w/v/v) and applied to the gel system of Takayama, et al., (1966), are shown in Figure 2.

SDS Systems

Figures 3 and 4 show the electropherograms obtained when the pellet fractions were applied to 10% and 5% SDS gels and stained with coomassie blue to show proteins. Figure 5 is the pattern obtained when the pellet fractions are stained for carbohydrate by the periodate-Schiff method after being assayed on 10% gels.

Figures 6 and 7 show the patterns obtained when the supernatant fractions were stained for protein after being applied to 10% and 5% gels. Figure 8 is the pattern obtained when the supernatant fractions are applied to 10% gels and stained for carbohydrate.

Figure 9 compares the patterns obtained for fractions 1-P and 5-P on a 10% gel stained for protein.

TABLE I. Recovery of fat globule membrane components in sequential supernatant and pellet fractions

Fraction	Protein[a]	Protein[b]	Lipid	Phospholipid
	. . . . . %			
Original[c]	100.0	----	100.0	100.0
1 - S	44.5	48.2	40.0	57.4
2 - S	11.9	12.9	12.5	16.5
3 - S	1.0	1.0	*	*
4 - S	1.0	1.0	*	*
5 - S	1.0	1.0	*	*
1 - P	47.8	51.8	38.8	48.4
2 - P	35.8	38.8	20.9	30.9
3 - P	34.9	37.8	19.7	29.9
4 - P	34.0	36.9	14.0	25.0
5 - P	33.1	35.9	13.5	26.1

(a) Percent recovery of protein with the original sample as 100.0%

(b) Percent recovery when the 7.7% loss of protein due to the fluff is disregarded

(c) Original material is freshly prepared buttermilk

(*) Insufficient sample for meaningful assay

TABLE II. Distribution of fat globule membrane components in sequential supernatant and pellet fractions (expressed as percentage)

Fraction	Protein	Lipid	Hexose	Sialic Acid	Hexosamine	CHO + Protein	Total
				%			
0	37.02	53.00	1.54	0.55	1.29	40.37	93.40
1-S	33.35	57.25	1.33	0.63	1.42	36.73	93.98
2-S	18.81	72.88	0.94	0.38	1.72	21.85	94.73
1-P	38.09	51.88	2.01	0.76	1.98	42.84	94.72
2-P	46.02	43.95	1.75	0.76	1.92	50.43	94.40
3-P	47.03	42.96	1.51	0.77	1.94	51.25	94.21
4-P	54.26	35.73	1.60	0.73	1.78	58.37	94.10
5-P	54.62	35.36	1.63	0.70	1.55	58.50	93.86

TABLE III. Distribution of fat globule membrane components in sequential supernatant and pellet fractions (expressed as component ratios)

Fraction	Protein / Total	Hexose / Protein	Sialic acid / Protein	Hexosamine / Protein	CHO / Protein	Phospholipid / Protein	Protein / Lipid	Phospholipid / Lipid
0	0.404	0.038	0.014	0.032	0.083	0.308	0.762	0.215
1-S	0.367	0.036	0.017	0.043	0.092	0.397	0.642	0.313
2-S	0.219	0.043	0.017	0.079	0.139	0.426	0.300	0.199
1-P	0.428	0.047	0.018	0.046	0.111	0.311	0.826	0.268
2-P	0.504	0.035	0.015	0.038	0.088	0.266	1.147	0.317
3-P	0.513	0.300	0.015	0.038	0.082	0.262	1.193	0.327
4-P	0.584	0.027	0.013	0.031	0.070	0.227	1.634	0.389
5-P	0.585	0.028	0.012	0.027	0.066	0.243	1.654	0.416

TABLE IV. Molecular weights of the polypeptides of fat globule membrane pellet fractions as determined by SDS electrophoresis in 10% polyacrylamide gels[a]

Fraction				
1-P	2-P	3-P	4-P	5-P
138,000	138,000	138,000	138,000	138,00
123,000	123,000	123,000	123,000	
118,000	118,000	118,000		
			112,000	112,000
110,000	110,000	110,000		
107,000	107,000		107,000	107,000
90,000	90,000	90,000	88,000	88,000
		86,000		
82,000	82,000			
74,000	74,000		74,000	74,000
70,000	70,000	70,000		
66,000	66,000	63,000	66,000	63,000
				58,000
			54,000	54,000
46,000	48,000	48,000	46,000	48,000
39,000	39,000			
36,000				
28,000				
23,000	23,000	23,000	23,000	23,000
15,500	15,500	15,500	15,500	15,500
13,500	13,500	13,500		

[a] average of five determinations

TABLE V. Molecular weights of the polypeptides of fat globule membrane pellet fractions as determined by SDS electrophoresis in 5% polyacrylamide gels [a]

Fraction				
1-P	2-P	3-P	4-P	5-P
208,000	208,000	208,000	208,000	208,000
				174,000
151,000	153,000	153,000	153,000	
140,000	140,000	140,000	140,000	
			133,000	133,000
			123,000	123,000
108,000	108,000	108,000	108,000	108,000
90,000	90,000	90,000		
			86,000	86,000
82,000	82,000	82,000	82,000	82,000
60,000	64,000	63,000	63,000	60,000
46,000	46,000	48,000	48,000	46,000
39,000	39,000	39,000	39,000	39,000
30,000	30,000	30,000	30,000	30,000
22,000	23,000	23,000	23,000	23,000

[a] average of five determinations

TABLE VI. Molecular weights of the polypeptides of fat globule membrane supernatant fractions as determined by SDS electrophoresis in 10% polyacrylamide gels[a]

Fraction					
0	1-S	2-S	3-S	4-S	5-S
123,000	123,000	123,000			
118,000	118,000	118,000	118,000	118,000	118,000
90,000	90,000	90,000	96,000	96,000	96,000
80,000	80,000	80,000			
76,000	74,000	74,000			
63,000	60,000	60,000			
56,000					
	48,000	48,000			
44,000	43,000	43,000			
40,000	40,000				
35,000	35,000				
32,000	32,000				
29,000					
26,000					
23,000	23,000				
17,000	18,000				
14,000	15,000				

[a] average of five determinations

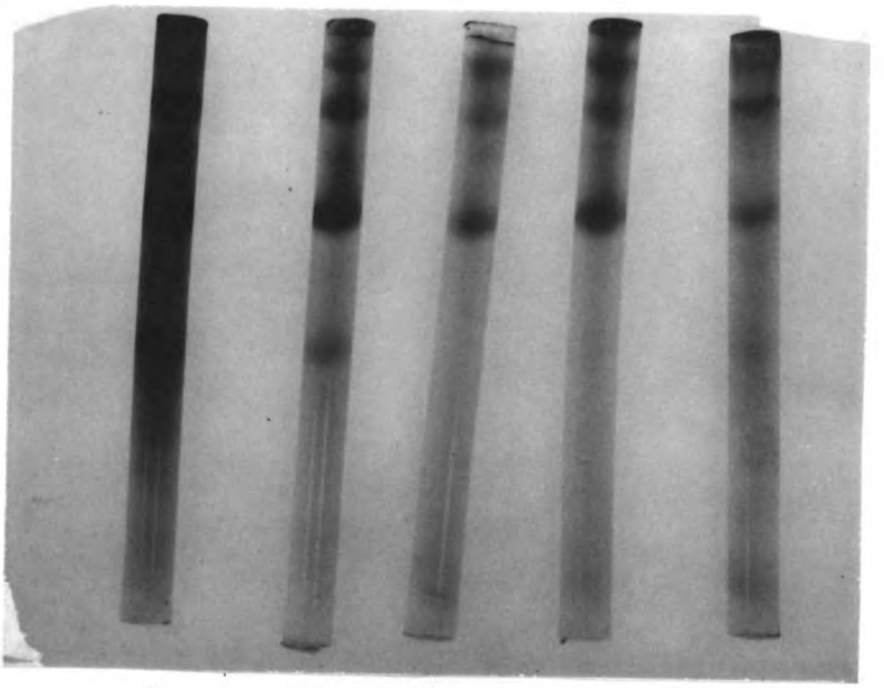
TABLE VII. Molecular weights of the polypeptides of fat globule membrane supernatant fractions as determined by SDS electrophoresis in 5% polyacrylamide gels[a]

Fraction					
0	1-S	2-S	3-S	4-S	5-S
153,000	153,000	153,000			
140,000	140,000	140,000	140,000	140,000	140,000
108,000	108,000	108,000	108,000	108,000	108,000
90,000	90,000	90,000			
81,000	82,000	80,000			
75,000					
60,000	60,000	60,000			
57,000					
48,000	48,000	48,000			
39,000	37,000				
32,000	32,000				
23,000	23,000				

[a] average of five determinations

Figure 1.--Electrophoretograms of five membrane fractions after solubilization and electrophoresis according to the method of Takayama, et al.

Figure 2.--Polyacrylamide SDS gel electrophoretograms [illegible]


0
15
2S
1P
5P
FRACTION

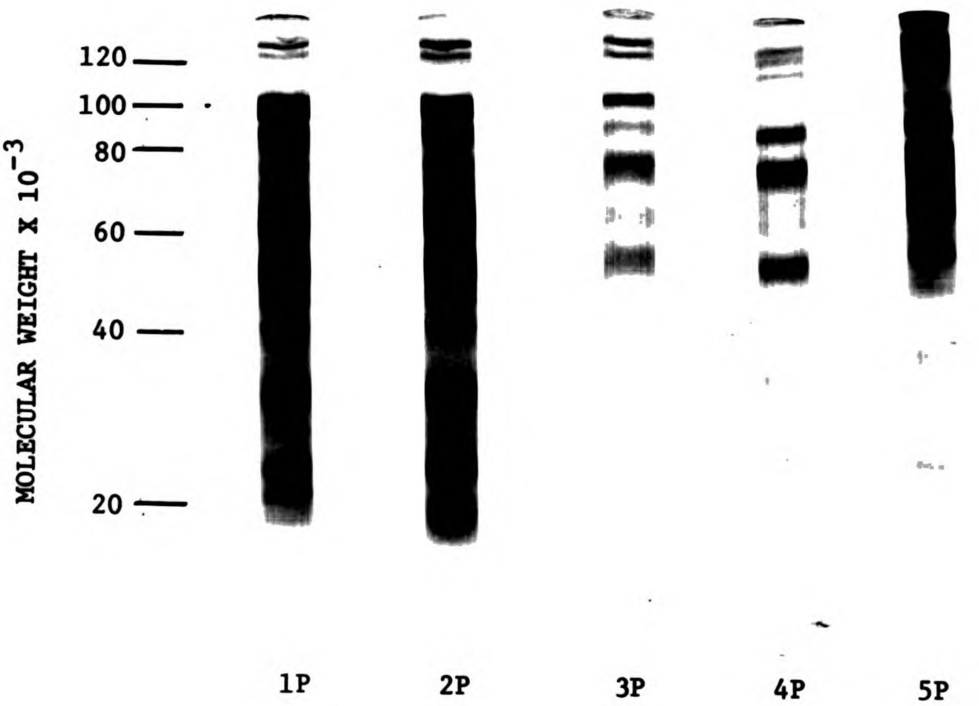
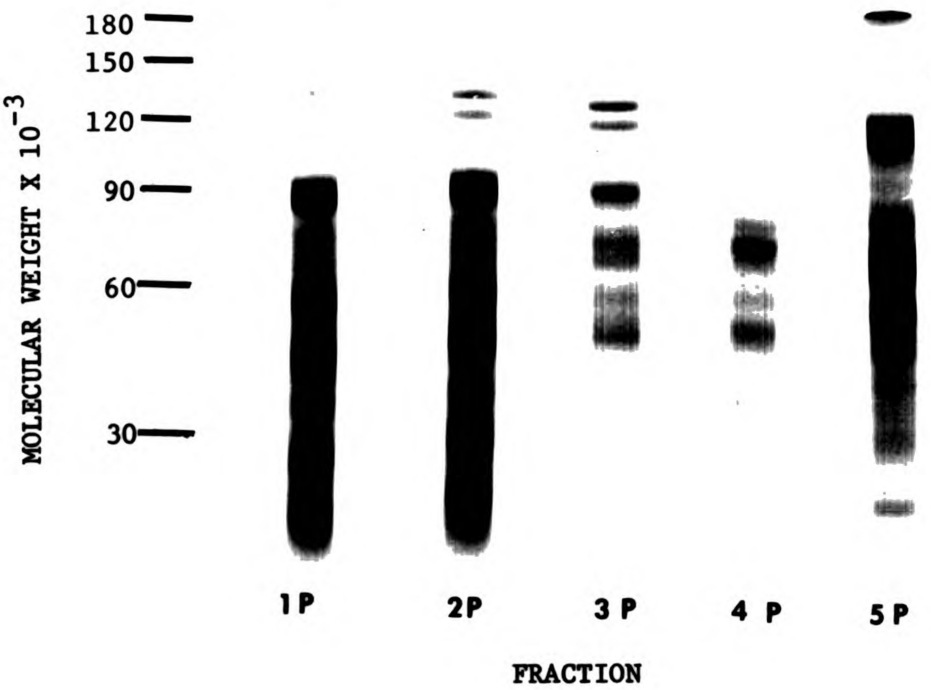

MOLECULAR WEIGHT X 10^{-3}
120
100
80
60
40
20
1P
2P
3P
4P
5P

Figure 4.--5% polyacrylamide-SDS gel electrophoretograms of the membrane pellet fractions stained for protein.

Figure 5.--10% polyacrylamide-SDS gel electrophoretograms of the membrane pellet fractions stained for carbohydrate.


MOLECULAR WEIGHT X 10^{-3}
180
150
120
90
60
30
1P
2P
3P
4P
5P
FRACTION

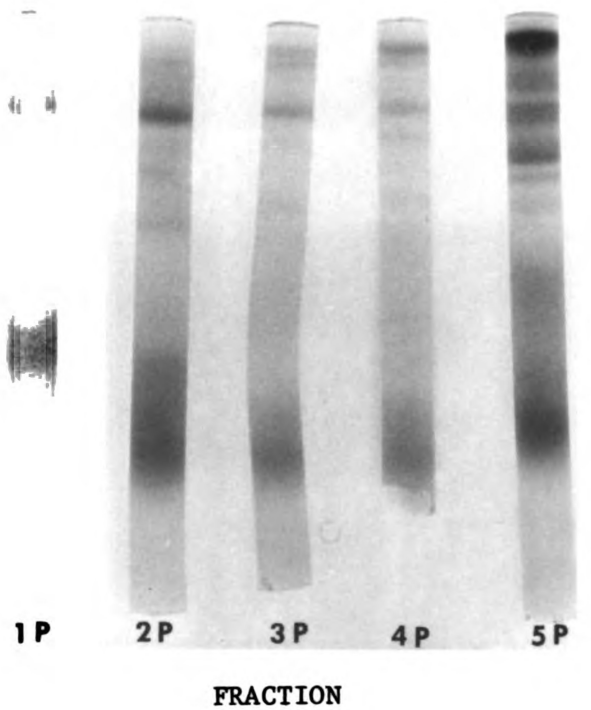
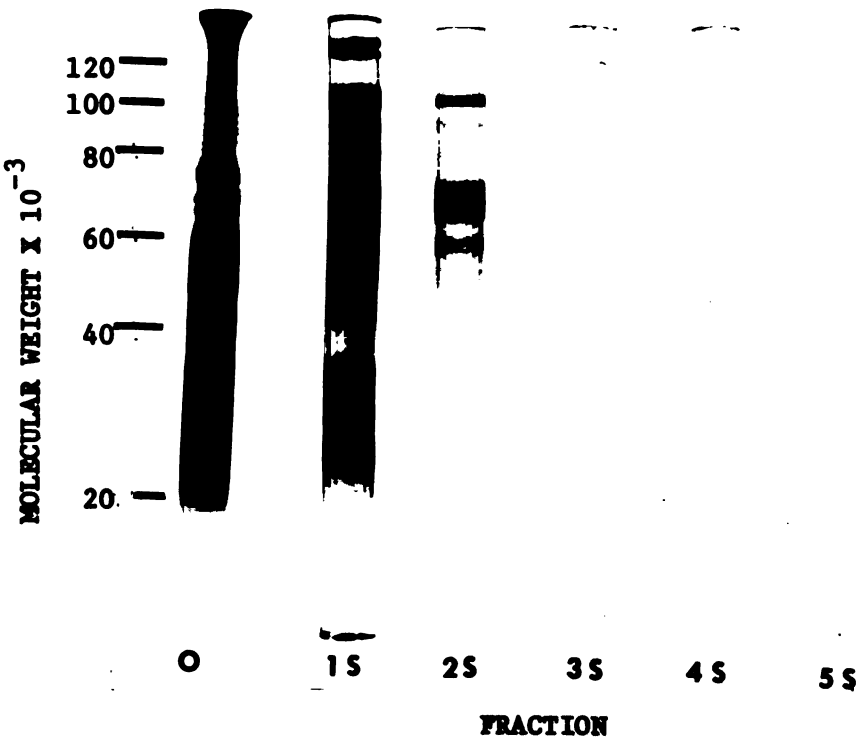

1P
2P
3P
4P
5P
FRACTION

Figure 6.--10% polyacrylamide-SDS gel electrophoretograms of the membrane supernatant fractions stained for protein.

Figure 7.--5% polyacrylamide-SDS gel electrophoretograms of the membrane supernatant fractions stained for protein.


MOLECULAR WEIGHT X 10^{-3}
120
100
80
60
40
20
0
1S
2S
3S
4S
5S
FRACTION

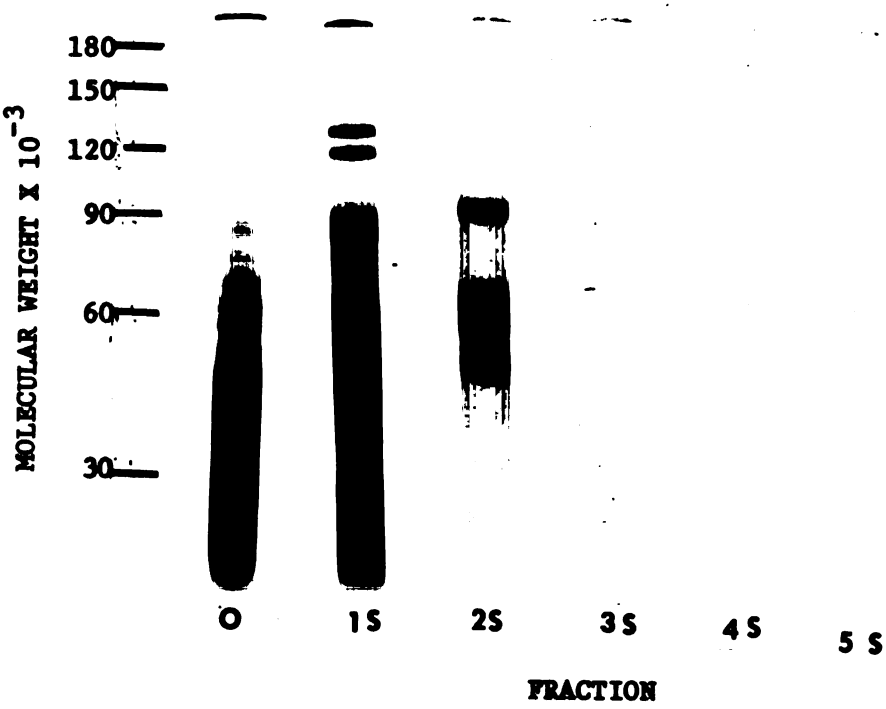
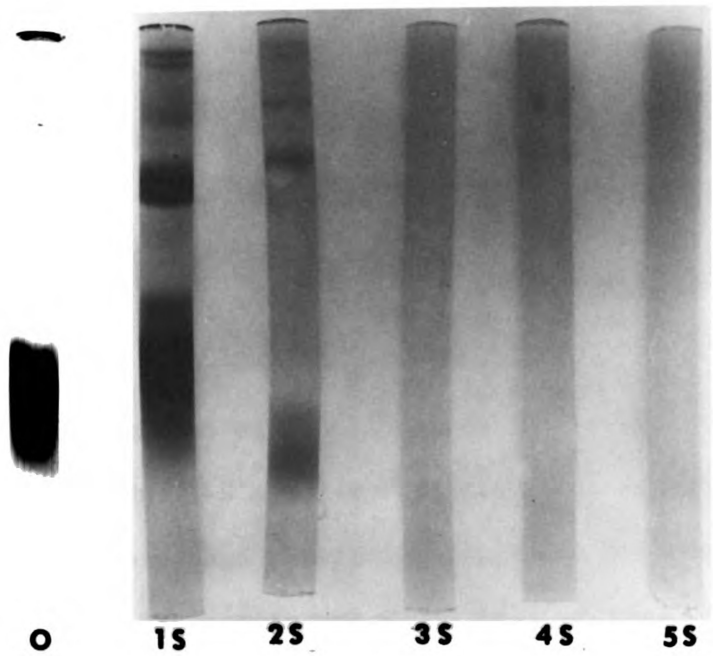

MOLECULAR WEIGHT X 10^{-3}
180
150
120
90
60
30
0
1S
2S
3S
4S
5S
FRACTION

Figure 8.--10% polyacrylamide-SDS gel electrophoretograms of the membrane supernatant fractions stained for carbohydrate.

Figure 9.--10% polyacrylamide-SDS gel electrophoretograms of pellet fractions 1-P and 5-P stained for protein.


O
1S
2S
3S
4S
5S
FRACTION


1P
5P
FRACTION

DISCUSSION

Distribution of Protein, Lipid and Phospholipid Between Membrane Fractions

Centrifugation of the original buttermilk material made to 0.6 M KCl to yield fractions 1-S and 1-P resulted in an almost even distribution of lipid, protein, and phospholipid between the two fractions. There was a 7.7% loss of protein and 21.1% loss of lipid following this preparative step which is attributed to the discarding of the flotation layer formed during centrifugation. A similar layer was reported by Swope (1968) who speculated that this material, high in lipid and low in protein, might be analogous to the chylomicron fraction of plasma lipoproteins.

Resuspension of the pelleted material with subsequent recentrifugation resulted in 25.10% of the protein, 46.13% of the lipid and 36.16% of the phospholipid of fraction 1-P being recovered in fraction 2-S. The amount of material lost to the flotation layer was 0.21% of the protein, 13.92% of the lipid, and 2.07% of the phospholipid.

It is doubtful that resuspension of fraction 1-P with 0.6 M KCl resulted in the solubilization of all the material that was found in fraction 2-S. More probably a large portion of this material was actually low-density lipoprotein that was pelleted in the first centrifugation, being trapped by the higher density material.

All further resuspensions and recentrifugations of the pellet fractions resulted in a solublization of less than 1% of the original

membrane protein which lends credence to the theory that the large amount of material present in fraction 2-S was not solely a result of solubilization. The low yields of fractions 3-S through 5-S negated meaningful chemicals analysis of these fractions.

Data in Table II show that the loss of lipid from each pellet fraction is much greater than the loss of protein. This observation indicates that the increasing insolubility of sequential pellet fractions results from the loss of lipid material.

Composition of Membrane Fractions

Pellets

The data in Table III represents the chemical composition of the supernatant and pellet fractions of the membrane material expressed as component ratios.

The protein and lipid contents of the five pellet fractions are inversely related to each other. Whereas the protein content in pellets 1-P to 5-P increases from 42.84% to 58.50%, the protein to lipid ratio increases from 0.83 to 1.65.

Closer examination of the data pertaining to lipid concentrations reveals that the ratio of phospholipid to total lipid increased from 0.27 for pellet 1-P to 0.47 for pellet 5-P, indicating that phospholipids are being selectively retained by these fractions. That some phospholipids are being lost can be seen by the decrease in the phospholipid to protein ratio between fractions 1-P and 5-P, viz., from 0.31 to 0.24.

The net effect is a gradual erosion of protein, a greater erosion of phospholipid and a much greater erosion of lipid from the pellet fractions as the extraction with KCl progresses. These data are shown graphically in Figures 10 and 11.

The hexose content of the pellets gradually declined from 2.01% of the total weight for pellet 1-P to 1.6% of the total weight for pellet 4-P. The loss of hexose from pellet 1-P to pellet 5-P represents a change of 18.91% of the total hexose. The change in the hexose to protein ratio, however, is much more dramatic. The ratio for pellet 1-P is 0.047 and 0.028 for pellet 5-P; a net change of 40.43%. In this case, as in all comparisons of carbohydrate to protein content, the actual loss of hexose is much less than is the decrease in the ratio of hexose to protein. This can be explained by the observation that the loss of lipid material is greater at each step than is the loss of carbohydrate. The loss of lipid increases the relative concentration of protein so that as the carbohydrate is decreasing by about 20% the protein concentration in the pellet is increasing by approximately 37%. The net effect is a dramatic decrease in the hexose to protein ratio.

The above relationship is most readily seen when the data for sialic acid are examined. The total percentage loss is from 0.76% for pellet 1-P to 0.70% for pellet 5-P or a loss of 8%. The ratio of sialic acid to protein decreases from 0.018 for pellet 1-P to 0.012 for pellet 5-P; a loss of 33%. The almost constant content of sialic acid between pellet fractions when compared to the ratio of sialic acid to protein for these pellets indicates that sialic acid containing proteins are lost from the pellet fractions.

The loss of hexosamine from the pellet fractions is noteworthy. The absolute amount of hexosamine remains almost constant for pellets 1-P, 2-P, and 3-P. There is a large loss between pellets 3-P and 4-P, and between 4-P and 5-P. The overall result is that from pellet 1-P to pellet 5-P the total loss of hexosamine and the ratios of hexosamine

to protein give values that are within experimental error of those obtained for hexose.

In terms of toal carbohydrate the change in percent of the total pellet from pellet 1-P to pellet 5-P is from 4.75 to 3.88%; a loss of 18%. The ratio of carbohydrate to protein changes from 0.111 to 0.066 for a loss of 40.5%. Again the change in toal carbohydrate to protein ratio is affected much more by the relative increase in protein concentration in sequential pellet fractions than by the actual loss of carbohydrate material. Restated, the loss of lipid from each pellet fraction is much greater than is the loss of carbohydrate. The loss of carbohydrate from pellet 2-P through pellet 5-P is linear within experimental error while the loss of lipid is stepwise.

The change in the ratio of carbohydrate to protein is stepwise in a manner closely following the decrease in lipid, indicating further that the major change in the composition of the pellet fractions is the loss of lipid material.

Supernatants

The first supernatant material is similar to the first pellet fraction, being lower in protein and carbohydrate than the first pellet and higher in lipid material. The main difference is the increase in the ratio of phospholipid to lipid.

The second supernatant material is characteristically different from other fractions, being high in lipid and low in protein with a protein to lipid ratio of 0.300. The hexose to protein and sialic acid to protein ratios are similar to fraction 1-P. The greatest difference is in the hexosamine to protein ratio of 0.079 compared to 0.046 for pellet 1-P, the fraction with the next highest ratio.

The phospholipid to lipid ratio of fraction 2-S of 0.199 is the lowest of all fractions examined while the phospholipid to protein ratio of 0.426 is the highest of any fraction. The total carbohydrate content of this fraction is 13.9%, a value comparable to the 15.0% estimated for a pure glycoprotein isolated from a soluble membrane protein by Swope, et al. (1968).

In general, from these data, certain trends can be seen in the composition of the membrane fractions as related to their insolubility. The most significant variables are the ratios of protein to lipid and phospholipid to protein.

In all cases, the more soluble fractions have a lower protein to lipid ratio and a higher phospholipid to protein ratio than do the more insoluble fractions.

Molecular Weights of Membrane Proteins

The molecular weights obtained for the various membrane fractions are listed in Tables III, IV, V, VI. The values listed represent an average of five determinations. If a component failed to appear on at least three to five replicates, it was not included in the total.

The tables list the molecular weights obtained in decreasing size for each fraction at a particular gel concentration. An empty space indicates that a band at that molecular weight failed to appear on at least three of the electropherograms obtained for that fraction.

While the molecular weight data obtained are informative, caution must be exercised in the interpretation of these results. It should be noted that the molecular weights derived from the two gel concentrations are in reasonable agreement between the range of approximately 55,000 to 110,000. Values outside of this range may vary by as much as 50%

for the same band at different gel concentrations and cannot be assured to be accurate until agreement on their molecular weight is achieved at two different gel concentrations (Rodband and Chramback, 1971). Even to consider the values within the region of agreement between the two gel concentrations as unequivocally accurate values can not be justified. The basic assumption for the accuracy of molecular weight determinations on SDS gels is that every protein binds the same amount of SDS and that separation is due to size alone (Weber and Kuter, 1971). That all proteins do not bind the same amount of SDS has been shown by Kogen, et al. (1972). They found that under optimum conditions elastin binds only 0.5 g of SDS per g of protein as compared to the assumed value of 1.5 g/g. Some, if not all, membrane proteins may vary from the ideal as was noted by Carraway and Koblyka (1970) who worked with erythrocyte membrane proteins. They stated that their results suggested that very hydrophobic proteins may show non-ideal behavior in SDS gels when compared to molecular weight standards. Another problem associated with making reliable molecular weight estimates for proteins in a complex system is that the relative mobility of a given band is governed by its concentration (Fairbanks, et al., 1971). When large amounts of sample are applied in order to visualize minor components the higher concentration of the major components increases their mobility as well as the mobilities of minor components that travel just ahead of them. Thus, a broad major compnent band may cover an area on a gel corresponding to a molecular weight of 20,000 or more. Always measuring the leading edge of the band does not completely standardize the method if more than one concentration of the sample is used to measure the relative mobilities. For these reasons, to consider the values listed in Tables IV through VII

the actual molecular weights of the membrane proteins would be open to severe criticism. True molecular weight values cannot be determined until each fraction is isolated in a pure enough form to allow for a more rigorous analysis.

The values obtained, however, are fairly reproducible for a given fraction at a given gel concentration. For this reason they may justifiably be used as points of reference to observe changes in the electrophoretic patterns obtained between fractions. Thus, a given band will be referred to by its apparent molecular weight in the indicated gel system.

Molecular weight estimates are not included for the periodate-Schiff reagent positive bands for two reasons:

1. Glycoproteins have been shown to bind varying degrees of SDS and their molecular weights vary greatly between different gel concentrations (Butscher, 1971).
2. The periodate-Schiff reagent positive bands were, in most cases, very diffuse which made the determination of a reproducible value for their relative mobilities precarious.

The diffuse characteristic of these bands is attributalbe to the employment of a dialysis step prior to staining to prevent adventitious dye binding to the SDS- protein complexes (Glossman and Neville, 1971). This requires prolonged soaking of the gels and probably allows for some diffusion of the glycoproteins.

Gel Electrophoretic Patterns Of Membrane Proteins

Acid Gels

Figure 2 shows the electrophoretic patterns of fractions 0, 1-S, 2-S, 1-P, and 5-P on the acid gel system of Takayama, et al. (1966).

An amount of each sample containing 5 mg of protein was dissolved in 1 ml of the phenol, acetic acid, water system as previously described. Twenty five 1 of each sample was applied to each gel. The system works well for the soluble fractions. However, the more insoluble fractions form a thick gel rather than a solution and further addition of solvent does not disperse the gel. This characteristic was noted by Osborn (1966) when a similar phenol containing system was used to solublize the membrane and envelope of various strains of Salmonella. This gelation characteristic makes representative sampling of the mixture uncertain and tends to impede a portion of the material from entering the gel. This phenomenon is shown in Figure 2 by the increasing amount of material that does not enter the gel and by the decreasing intensity of the bands that do enter when fractions of increasing insolubility were examined. For these reasons this system was considered unsatisfactory for comparing the membrane fractions and a different solublizing system was utilized.

SDS Gels

Figure 3 shows the electrophoretic patterns obtained from fractions 1-P through 5-P applied to a 10% SDS gel and stained with coomassie blue. The amount of material applied to each gel was varied in an attempt to

show as many of the bands as possible. Attempts to employ densitometer tracings of the electropherograms failed because many of the bands migrated in close proximity, thus, negating base line readings between zones. Longer gels offered no improvement in separations. The high ionic strength of the buffer system was reflected in slow migration rates of the proteins. Increased gel length requires greater running time, obstructing any increase in resolution becasue of increased diffusion. For these reasons, comparisons of the relative contribution that each zone makes to the total pattern of a given fraction must be estimated visually.

Examination of the electropherograms shown in Figure 3 indicates that changes are occuring in the protein distribution of successive membrane pellet fractions. One zone in the first three pellet fractions possessed an estimated weight of 90,000. With increasing insolubility of the pellet fraction, this band decreased in intensity until in fractions 4-P and 5-P it represents a minor component. Similarly, the major bands in fraction 1-P, with molecular weights of 66,000 and 46,000, decreased in intensity with successive extractions. Also, there seems to be a decrease in the zone representing a molecular weight of 15,500, and the zone with a molecular weight of 13,500 is on longer visible beyond fraction 3-P. These alterations in the pellet composition appear to be balanced by an increase in the intensitives of the zones attributable to molecular weights of 74,000, 112,000 and 138,000 in fraction 5-P. Fraction 5-P is the only specimen containing appreciable amounts of material unable to enter the gel. The electropherograms in Figure 4 indicate that pellet fractions separated on 5% gels show similar changes in protein distribution. The principal difference being in the values estimated for the molecular weights obtained for the zones at both

extremities of the gel.

The extreme change in the protein patterns between fractions 1-P and 5-P are apparent in Figure 9 which compares these two fractions on 10% gels.

There are two possible explanations for the changes that occur in the electrophoretic patterns of the pellet fractions:

1. That extraxtion with KCl is selectively removing some protein components and therefore causing an increase in the residual components.
2. That extraction with KCl is promoting a reorganization of the proteins in the system.

Analytical data overwhelmingly support the second possibility. The amount of protein lost to the supernatant fraction from fraction 1-P is low and in subsequent extractions the loss is neglibible. Concurrently, there is a notable change in the protein to lipid and phospholipid to protein ratios of these fractions.

As lipid is lost from successive pellets, the residual protein tends to aggregate as is evidenced by the decrease in intensity of low molecular weight and a concurrent increase in the intensity of the large molecular weight species comprising fractions 1-P to 5-P. This apparent aggregation suggests that the hydrophobic groups of the protein molecules are involved.

Figure 5 represents the electrophoretic patterns of the pellet fractions applied to 10% SDS gels and stained for carbohydrate. The patterns indicate that the large molecular weight proteins contain carbohydrate while the smaller ones do not. As was previously noted, it was impossible to correlate the carbohydrate-staining polypeptides with the coomassie blue-staining species. Comparisons of the patterns in Figures

3 and 5, however, suggest that the major zones of the pellet fractions contain carbohydrate. In the first pellet fraction (1-P), the most intensely stained zone corresponds to the 90,000 molecular weight zone. In the final pellet, (5-P), the molecular weight of the most intense zones were estimated at 74,000 and 112,000. The very diffuse zone at the bottom of each gel possesses characteristics of a glycolipid (Leonard, 1970).

The reason that the lower molecular weight species did not stain for carbohydrate may be that they were removed from the gels during the long soaking period required to remove the SDS. Whatever the cause, these gels add qualitative proof to the contention that at least some of the membrane proteins are glycoproteins.

The electrophoretic patterns of the original material and of fractions 1-S through 5-S on 10% SDS gels are shown in Figure 6. In general, the zones from the supernatant fractions are more distinct and show less distortion than do the zones of the pellet fractions. This is an indication of greater homogenity of the components and of less association between different proteins (Nichol, et al., 1964). Fraction 3-S through 5-S show a very faint zone with a molecular weight of 118,000 and, when larger amounts of material are placed on the gels, another fainter zone with a molecular weight of 96,000. Thus, it appears that the small amount of protein that is being solublized by KCl extraction following the second pellet is the 118,000 molecular weight component. It is of interest to note that one of the major components of fraction 5-P, the most insoluble fraction, showed a molecular weight of 112,000.

The differences in patterns between fractions 0 and 1-S are noteworthy. Fraction 0 is richest in the lower molecular weight species,

showing two main zones of molecular weights of 35,000 and 32,000. Fraction 1-S is rich in the low molecular weight componenent, viz., 15,000, but has three main zones with molecular weights of 48,000, 60,000 and 90,000. Immediately apparent from these patterns is the shift in the amounts of the higher molecular weight materials between fractions 0 and 1-S. A re-examination of the pattern of fraction 1-P indicates that it is also richer in the higher molecular weight components, indicating that the original treatment of the membrane material caused a shift towards the higher molecular weight species. This condition may have resulted from hydrophobic aggregation occuring concomitantly with the loss of lipid material. This phenomenon is common in other lipoprotein systems much as blood plasma (Scanu, 1967), egg yolk (Cook and Martin, 1969), and mitochondrial structured protein (Green and Fleischer, 1963).

The 2-S fraction seems to consist of two main zones that are also the main zones of fraction 1-S. They possess molecular weights of 60,000 and 90,000.

Figure 7, showing electropherograms of the supernatant fractions run on 5% gels, illustrates the same shift towards higher molecular weight species between fractions 0 and 1-S.

The carbohydrate-containing proteins in the supernatant fractions are shown in Figure 8. Again, the high molecular weight species stain more intensely for carbohydrate than do the lower molecular weight species. A notable exception is the low molecular weight zone in fraction 1-S found just before the glycolipid smear. It should also be noted that in fraction 2-S the top two zones which are barely visible in Figures 6 and 7 have become more prominant, indicating that these proteins are rich in carbohydrate.

The funnel shaped smear that is seen to extend over the top one-fourth of the gel for fraction 0 was always present when this fraction was electrophoresed. Before staining it could be seen as an opalescent area and is probably related to the higher lipid content of this fraction.

It should also be noted that fraction 3-S through 5-S, when stained for carbohydrate, did not contain the glycolipid smear that was so prominant in the previous fractions.

At first, consideration was given to the fact that the densities of the fractions were increased to aid in application to the gels by the addition of sucrose and that this smear might represent an artifact caused by such additions. Sucrose was added to fractions 3-S through 5-S, however, and the absence of any smear indicated that the soaking period had effectively removed any free sucrose that might be present.

The electrophoretic patterns raise many questions that can not at this time be answered. The principal problem being to determine to what extent the distribution of proteins seen on the gels is related to their distribution in the native system. It remains to be determined how many of the zones are single proteins and how many, if any, are the result of aggregation of similar or different protein species. Also to be determined is the effect that high concentrations of mercaptoethanol incorporated into the solublizing system has on the protein patterns observed. Normally, it would be expected that inter-chain disulfide bonds would be broken, yielding more subunits than in the native state. Leonard (1970) states, however, that while the use of mercaptoethanol is necessary to achieve complete solublization of erythrocyte membranes, it does not function by breaking disulfide bonds. It is possible that the interaction between

the double bonds of the lipid hydrocarbon chains and sulfhydryl groups of proteins may be broken by the mercaptoethanol (Robinson, 1966). It has not been determined if these situations apply to the proteins of the fat globule membrane.

Elucidation of these questions can only be achieved by studies with the isolated proteins of the membrane system. Then, their true molecular weights and amino acid composition can be compared. Until such data are obtained, the questions raised will remain unanswered.

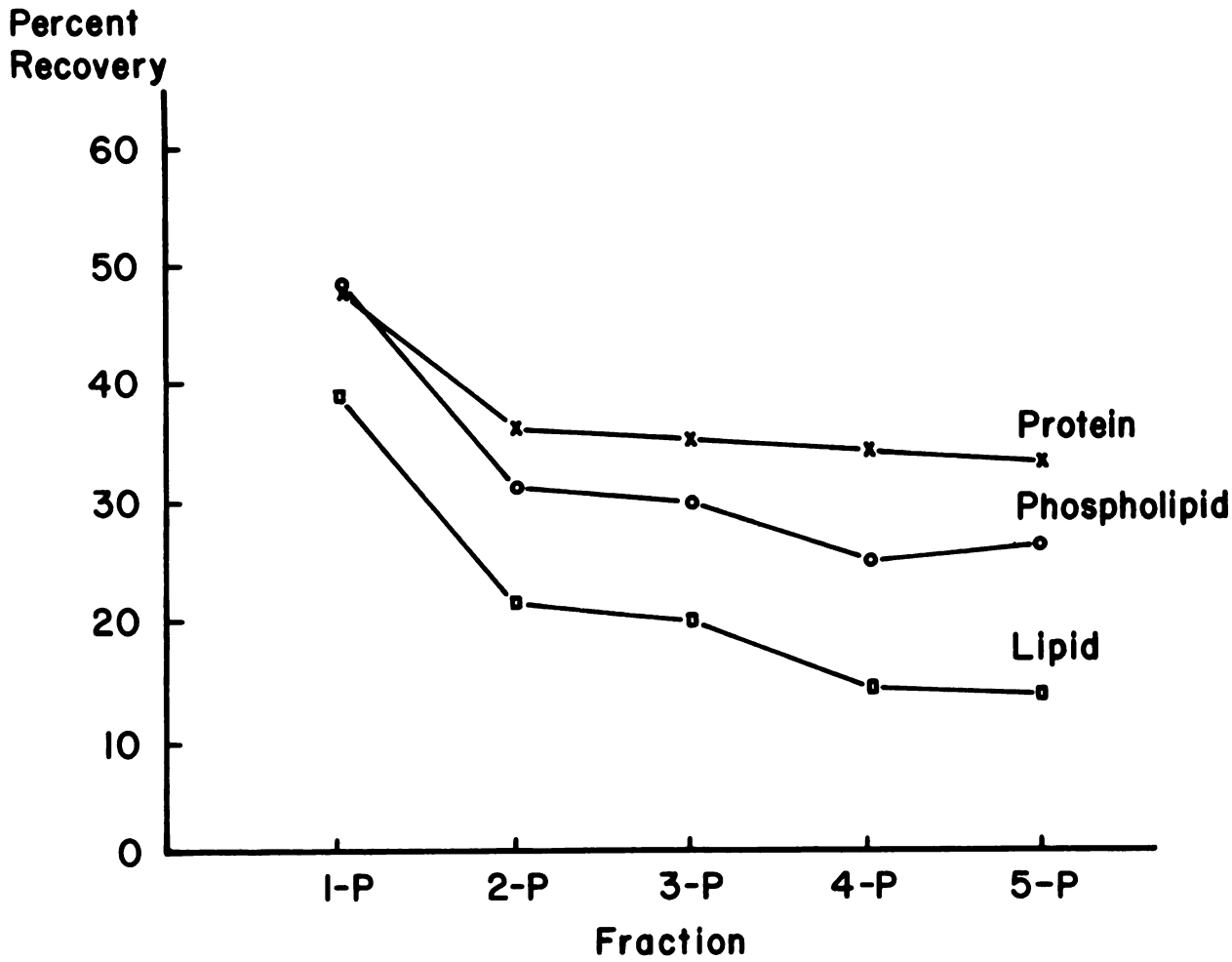


Figure 10. Recovery of milk fat globule membrane components in the pellet fractions.

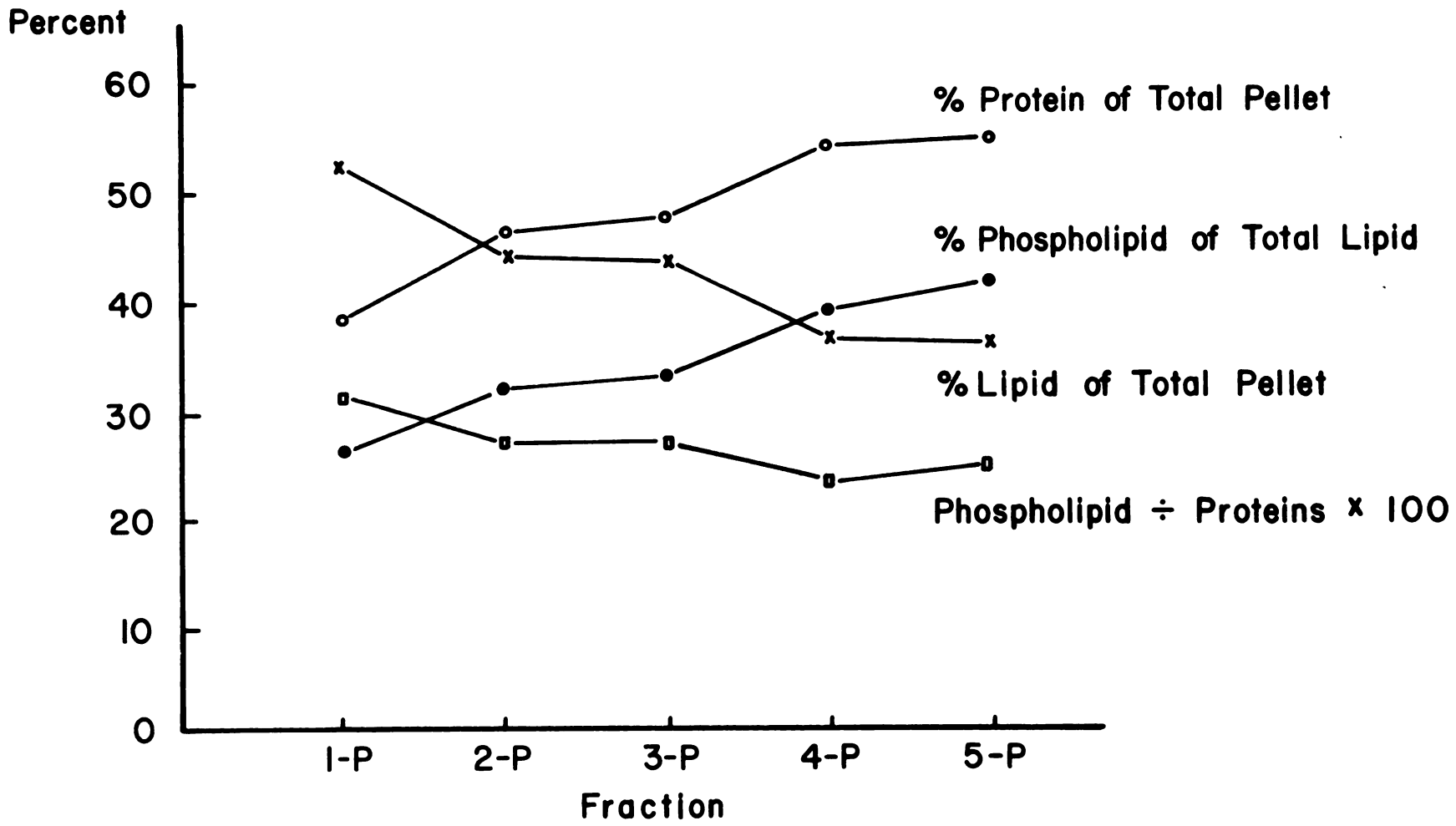


Figure 11. Composition of milk fat globule membrane components in the pellet fractions.

CONCLUSIONS

1. Sequential extraction of milk fat globule membrane material with 0.6 M KCl and subsequent centrifugation of the extracted material yielded five supernatant and five pellet fractions of varying solubilities.

2. As the fractions decrease in solubility, the protein to lipid ratio increases and the phospholipid to protein ratio decreases.

3. The total carbohydrate content and the ratio of carbohydrate to protein decrease with decreasing solubility of the protein species.

4. The most soluble fraction, 2-S, has the highest ratios of phospholipid to protein, hexosamine to protein and the lowest protein to lipid ratio.

5. The removal of lipid by extraction with KCl seems to be the main cause of decreased solubility.

6. There seems to be an aggregation of low molecular weight proteins to form higher molecular weight species as the degree of insolubility increases.

7. The larger molecular weight proteins stain more intensely for carbohydrate than do the lower molecular weight proteins.

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Tabel A1. Some important chemicals used in this study and their sources.

Chemical	Source
Schiff Reagent	Fisher
Mannose	
Acetylacetone	
2-mercapotethanol	
Urea	
SDS	
2-thiobarbituric acid	Eastman Organic Chemicals
N,N,N',N',-tetramethylethylenediamine (TEMED)	
Acrylamide	
N,N'methylenebisacrylamide	
Sodium azide	
TRIS-hydroxymethyl aminomethane (Sigma 121, Trisma base and Sigma 7-9)	Sigma
Galactosamine	Nutritional Biochemicals
N-acetylneuramic acid	Calbiochem
Galactose	Phanstiehl Laboratories, Inc.