STRUCTURE OF THE MILK FAT GLOBULE MEMBRANE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Marvin Paul Thompson

1960

This is to certify that the

thesis entitled

Structure of the Milk Fat Globule Membrane

presented by

Marvin Paul Thompson

has been accepted towards fulfillment
of the requirements for

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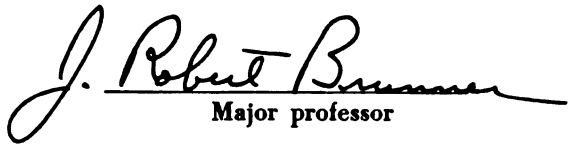

Major professor

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STRUCTURE OF THE MILK FAT GLOBULE MEMBRANE

By

Marvin Paul Thompson

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

Department of Food Science

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ABSTRACT Marvin P. Thompson

This research study involved a comprehensive evaluation of the protein-lipid structure of the milk fat-globule membrane. Employed in such a study were ultracentrifugal, electrophoretic, silicic acid chromatography, and other valuable analytical techniques.

The lipid portion of the membrane is comprised mainly of triglycerides and phospholipids in a 2:1 ratio. The nature of the triglyceride, which appears to be essentially high melting lipid, was elucidated. Interestingly, the membrane is composed of carotenoids, a squalene-like compound, cholesterol and its esters, and mono-diglycerides in addition to triglycerides and phospholipids. The qualitative and quantitative evaluation of the lipid components depends, in part, on the method of their preparation.

By differential centrifugation, a lipoprotein fraction was isolated. Its lipid distribution, as opposed to the other fractions obtained by sedimentation, suggests that it is the outer layer of the membrane material. In conjunction with this protein, studies were performed on lipid-free soluble membrane-proteins and compared with soluble membrane-proteins previously reported in the literature. By virtue of the similarities of these fractions, it was concluded that all isolated soluble membrane-protein fractions were essentially identical differing only in the percentage of bound lipid.

ABSTRACT Marvin P. Thompson

Structurally, the data have shown the fat-globule membrane protein to be bound to the fat globule by means of a high-melting glyceride fraction. Additional information suggests that an insoluble protein fraction, low in lipid concentration, is intertwined between the high-melting triglyceride and the true lipoprotein.

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A THESIS

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DEDICATION

To my mother and father

AUTOBIOGRAPHY

I, Marvin Paul Thompson, Sr., was born of wonderful home on June 22, 1933, in Troy, New York. My primary education was received in Watervliet, Latham, and Waterford, New York, from September, 1938 to June, 1947. Secondary education was received in Waterford High School from September, 1947 to June, 1951.

Professionally, my college education began at the New York Institute of Agriculture, Cobleskill, New York, where I majored in Dairy Technology, and graduated in June, 1953 with a degree of Associate in Applied Science. During the same month I married Virginia B. Pollock who has been my faithful companion for these several years. From 1953 to 1956, I attended Kansas State University, majoring in Dairy Manufacturing, and in January, 1956 received the degree of Bachelor of Science. Kansas State became the first graduate school that I was fortunate enough to attend. Studying under Dr. Bill Rutz, I received the degree of Master of Science in Dairy Manufacturing in January, 1957. On September 25, 1956 my first daughter, Faith Ina, was born.

From 1957-1960, I had the extreme pleasure of studying under my good friend and superb researcher Dr. Bob Brunner, who was an inspiration in my life. Our mutual interests, discussions and yet absence of complacency made our relationship enjoyable. During the course of my study at Michigan State University my son, Marvin Paul Jr., and second daughter, Hope Diane, were born.

In November, 1960 I received the degree of Doctor of Philosophy, majoring in Food Science, with a thesis entitled, "Structure of the Milk Fat-Globule Membrane."

ACKNOWLEDGEMENTS

The author expresses his sincere gratitude to Dr. J. Robert Brunner, professor of Food Science, for his encouragement and motivating interest during the course of this study. His realistic philosophy, understanding, and sympathy are appreciated, not alone by this individual, but by all who know him.

Next, and never last, to my beloved wife, Virginia, and to my two lovely daughters and son, go my thanks for their sacrifices. And to my parents, to whom this thesis is dedicated, go thanks beyond words for their assistance.

To Dr. C. M. Stine, assistant professor of Food Science, go many thanks for contributing grossly to the lipid section of this thesis.

Lastly, this author is grateful to his fellow graduate students for their encouragement and willingness to contribute in any way possible during the course of this study.

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INTRODUCTION

Little doubt exists that a lipid-protein complex is present at the surface of the fat globules present in whole milk, and that this lipid complex serves a useful purpose in stabilizing the fat-in-oil emulsion. This lipid-protein complex, commonly referred to as "surface layers" or "membrane" material, is intricate enough to stagger the imagination of the most enthusiastic researcher. And yet, the fraction has been studied in relatively little detail.

Needless to say, the milk fat-globule membrane has been the subject of much controversy; nevertheless, there is considerable agreement that it is demulsified as a result of freezing, is involved in the production of "free fat" in whole milk powder, contributes to oxidation of milk products, and is affected by such processing procedures as pasteurization and homogenization. These phenomena make a study of the membrane imperative, a study which will contribute to the understanding of the basic properties of the material. Of academic interest is the "fine structure" of the membrane, including the proteins and lipids present, which contribute to the oil-in-water emulsion existing in milk. On the practical side of the question, an understanding of the nature of the membrane material could lead to processing procedures designed to produce more uniform milk products. For example, whole milk powder possesses a relatively poor dispersibility, in part due to the presence of hydrophobic free fat. Is it

possible then to stabilize the fat-globule membrane by the addition of a suitable stabilizer or by alteration of processing procedures to produce a more palatable food product? Such questions are yet open to research.

The present concepts of the structure of the milk fat-globule membrane lack exactness which can be obtained only by years of research. Thus, the research data contained in this thesis were obtained from the desire to basically study the lipid-protein complex of the milk fat-globule membrane, and to contribute to understanding of this complex. Research has been directed toward the qualitative and quantitative evaluation of membrane lipids, isolation and identification of protein fractions, and evaluating the nature of the protein-lipid complex of the membrane from literature data and data contained herein.

REVIEW OF LITERATURE

Historical

Several reviews of the literature have been made on the milk fat-globule membrane: Palmer (1944), Brunner (1952), King (1955) in an excellent book, Rosenberg (1957), and Jackson (1959). Thus, the author will dedicate this review of literature to the subject area treated in the thesis with the realization that historical literature is valuable in building present concepts.

The nature of the protein of the milk fat-globule membrane has been a question of controversy for about 70 years. Babcock (1889) theorized that the protein surrounding the membrane might, in reality, be a fibrin on the basis that creaming of milk and coagulation of blood were similar. Hekman (1923) apparently disproved this theory, but it is interesting to note that the coagulation of blood and the coagulation of the caseins is very similar. Storch, in 1897, postulated that the substances surrounding the membrane were mucin-like, and advanced the theory that these mucin-like substances contained carbohydrate. Thompson and Brunner (1959c) confirmed the "ancient" research of Storch and, indeed, the membrane is in part carbohydrate.

The question as to whether casein exists in the membrane is a problem requiring due thought. Abderhalden and Völtz (1909) reported that the protein was not casein; however, Titus et al. (1928) concluded that protein was quite similar to casein. Mulder and Menger (1958a)

appear convinced that casein, as well as albumin and globulin, are an intricate part of the fat-globule membrane but are loosely bound. Thus as he points out, washing procedures such as those employed by Rimpila and Palmer (1935) and Brunner et al. (1953a) are apt to remove the loosely bound proteins. If such washing conditions are justified, they are justified only on the basis that the tenaciously bound membrane (not the native membrane) is secured (Mulder and Menger, 1958a). Concurrent to Mulder's research, Sasaki and Koyama (1956) postulated that the membrane did not contain the caseins or beta-lactoglobulin, but did contain a part of the whey protein plus another protein. On the basis of amino acid compositional analyses on the whole membrane material prepared from washed cream by Hare et al. (1952) and Brunner et al. (1953a), and on fractionated soluble and insoluble membrane proteins by Herald and Brunner (1957), it appears unlikely that the membrane protein is casein in nature. Of course, amino acid analyses are insufficient data to completely answer the question.

"Haptein" isolated by Hattori (1925) was unlike any other known protein of milk on the basis of solubility, sulfur and cystine content. These characteristics of the protein permitted Hattori to suggest that the protein was "keratin-like." Sandelin (1941) isolated an ammonia insoluble protein from milk which he thought to be identical to the membrane protein. Herald and Brunner (1957) carefully studied an insoluble fraction from the fat-globule membrane which they obtained after ethanol-ether extraction of the membrane material. On the basis

of (a) insolubility in 0.02 M sodium chloride solution, (b) reactivity to agents commonly employed in disrupting disulfide bonds, (c) affinity for alkaline solution and (d) a 1:1 ratio of lysine to arginine, they tentatively classified the protein as a pseudokeratin. This research gave appreciable support to the presence of an insoluble protein not adequately recognized by other researchers.

In 1924, Palmer and Samuelson indicated that the substance stabilizing the fat-water emulsion in milk was a single globulin-like protein complexed with phospholipids. Furthermore, Rahm and Sharp (1928) considered that foaming of milk was due to "schaumstaff" which was thought to be a protein associated with the fat globules. Unfortunately, like much early research, tools were not available to classify "schaumstoff" or the substance in question. A considerable lapse of time became apparent before any precise identification of the proteins was illuminative. Brunner et al. (1953a) successfully extracted the lipid portion of the membrane using cold alcohol with subsequent ether extractions. In a succeeding publication, Brunner et al. (1953c) showed the membrane to contain as many as four electrophoretically discernible components. The fraction was shown to contain one principal sedimenting component with a sedimentation coefficient of 7.3, a result which suggested the protein to be "globulin" in nature (Brunner et al. 1953b), a suggestion which may have been premature.

Herald and Brunner (1957), as was previously stated, successfully fractionated membrane proteins, which were virtually lipid-free,

on the basis of their solubility in 0.02 M NaCl. The 0.02 M NaCl soluble fraction contained 11.10% nitrogen, 0.7% sulphur, 0.46% phosphorus, 7.06% ash, and gave a strong positive Molisch test for carbohydrate. Later, Brunner and Herald (1958) reported that this fraction contained 2-3 electrophoretic components depending upon the buffer system employed. Thompson and Brunner (1959) confirmed the presence of carbohydrate in this fraction, and these researchers consider the fraction to be, at least in part, glyco-protein.

It is important to note that all electrophoretic studies on the membrane proteins have been reported on lipid-free fractions. However, Sasaki and Koyama (1959) report that 2-3 lipoproteins exist when examined on paper electrophoresis. Their report is the first published attempt to examine the true membrane lipoproteins(s).

Before leaving this subject area, it is well to consider that several researchers have attempted to determine the amount of protein present in the surface layers. Rimpila and Palmer (1935) report 0.46 - 0.71 g. protein/100 g. fat, whereas Jenness and Palmer (1945b) give the value of 0.38 - 0.86 g. protein/100 g. fat. These results are certainly in good agreement. Hare et al. (1952) reported a decidedly low value of 0.026 g. protein/100 g. fat. More recent data, Herald and Brunner (1957), gave a protein content of 0.51 g./100 g. fat, whereas Mulder and Menger (1958a) calculated protein values

ranging from 0.1 - 3.0 g./100 g. fat. These later studies were performed on 50 experiments, and the authors felt that amounts up to 0.8 g. protein/100 g. fat may perhaps be low, but were of the opinion that this value was reasonably representative of the situation in normal milk.

The milk fat-globule membrane contains enzymes in addition to other proteins. As early as 1933, Kay and Graham studied the phosphatase enzyme associated with the membrane. In 1956, Zittle et al. examined the alkaline phosphatase and xanthine oxidase distribution in skimmilk and cream. Morton (1950) reported a whole galaxy of enzymes in membrane "microsomes," which included alkaline phosphatase, xanthine oxidase, diaphorase, DPN-cytochrome c, reductase, and even nucleic acids. Zittle et al. (1956) found only 0.5% nucleic acid associated with the membrane. Confirmation of the presence of alkaline phosphatase and xanthine oxidase was made on the soluble and insoluble membrane proteins by Herald and Brunner (1957) and Rosenberg (1958).

In addition to the protein fraction of the membrane, lipids of various classes exist. Early in the study of the membrane material, Palmer and Weise (1933) were the first to isolate a high-melting glyceride fraction, and later Rimpila and Palmer (1935) found the HMGF to be present in fat-globule membranes from artificial creams. Jenness and Palmer (1945a) further investigated this fraction which was ethanol insoluble at 24-25°C. and reported rather constant properties. Its low iodine value (5.0 - 7.1), saponification equivalent

(198.8 - 204), and high melting range (52-53°C.) suggested that this fraction was unique to the membrane. However, it was also reported that the HMGF was present in butterfat to the extent of 4.5% of the total lipid. Interestingly, these same researchers observed that butter plasma extract was exceedingly rich (37.4%) in HMGF. Rimpila and Palmer (1935) postulated the special strong affinity between the HMGF and the fatty acid residues of the phospholipids.

Patton and Keeney (1958) and Thompson et al. (1959a) studied the HMGF concurrently but independently of each other, and agreed that palmitic and stearic acids make up the primary fatty acid residues of the fraction. Patton and Keeney (1958) prepared the glycerides by acetone precipitation whereas Thompson et al. (1959a) prepared it by ethanol precipitation. Since Thompson et al. (1959a) found only traces of fatty acids in the ethanol supernatants from the crystallization steps, they suggested that the entire membrane triglyceride is HMGF.

Palmer and Weise (1933) again were forerunners in showing that phospholipids were associated with the membrane. The following year, Kurtz et al. (1934) isolated an ether-soluble lecithin-cephalin fraction (about 56% lecithin and 44% cephalin) which contained a remarkably high percentage (70.6%) of oleic acid. Rimpila and Palmer (1935) reported 0.17 - 0.29 g. phospholipid/100 g. fat, Heinemann (1939) reported 0.27 - 0.28 g. phospholipid/100 g. fat, and Jenness and Palmer (1945b) found 0.18 - 0.43 g./100 g. fat.

More recently, Rhodes and Lea (1958) and Smith and Jack (1959) have isolated and identified the phospholipids of bovine milk. In 1957, Mulder et al. (1957b) reported that 100 g. of fat globules retain an average of 600 mg. of phospholipids, and that 60% of the total phospholipids present in milk are present in the surface layers. The surface layers themselves contained 30% phospholipid. Thompson et al. (1959d) reported the presence of cephalin, lecithin and sphingomyelin in the membrane lipid as determined by silicic acid chromatography.

According to Kon et al. (1944) the carotenoids and cholesterol appear to be associated with the fat-globule membrane. White et al. (1954) observed that carotenoids and vitamin A were loosely bound to the membrane in layers of constant depth. The carotenoids were calculated to be concentrated in the membrane to the extent of 0.0645% as compared to 0.000476% on the interior of the globule. The corresponding values for vitamin A were 0.0483 and 0.000381% respectively.

The cholesterol content of milk has been reported to be 0.3 - 0.4 g/100 g. fat. Mulder and Zuidhof (1958b) report the mean figures for cholesterol content of milk fat, the milk fat globules, the surface layers and serum to be 286 ± 7 mg./100 g., 322 ± 9 mg./100 g., 36± 16 mg./100 g., and 2.2 ± 1.2 mg./100 g. fat, respectively. About 15% of the cholesterol present is esterified; these esters occurring in the surface layers and in the serum.

The earliest concepts of the structure of the milk fat-globule membrane were first presented by Rimpila and Palmer (1935), and

were discussed in more detail by Bird et al. (1937). For more specific details, the reader is referred to these publications. In order for the surface layers to be rigidly bound to the fat globule, a substance must exist which is capable of bonding the surface layers with the fat globule. The high-melting glyceride fraction could serve such a purpose since it possesses fatty acid residues of sufficient length to bind, by van der Waals attraction forces, with the lipoprotein layer. Such a bonding would occur preferentially through the phospholipid side chains. This was demonstrated by Jenness and Palmer (1945a) who observed that when butter melts a considerable portion of the HMGF is drawn into the butter plasma by the lipoprotein.

Concerning the depth of the phospholipid layer, King (1955) calculated that a monomolecular layer of radially oriented phospholipid molecules 2.2 mμ long at the surface of the milk fat-globules possessing a diameter of 3μ would correspond to 0.38 g. phospholipid/100 g. fat. Jenness and Palmer (1945b) stated that 15-22 μg. of phospholipid/100 sq. cm. of fat surface gave a thickness of 1.8 - 2.6 mμ for this layer or a length of 2.2 mμ for a radially oriented phospholipid molecule. Thus, it appears that the membrane contains an adequate concentration of phospholipid to sufficiently surface a 3-4 μ fat globule.

The remaining portion (or water layer) of the membrane is completed by the hydrophilic protein molecules whose ionizable groups serve to give the fat globule its dispersion properties. If, indeed, caseins and serum proteins are loosely adsorbed on the surface layers,

they would be at the extremities of the moiety comprising the complex. The role of the insoluble membrane protein is still obscure. This protein and other features of the membrane structure are contained in the text of this thesis, and will not be further pursued here.

Finally, a consideration of the origin of the fat-globule membrane should be made with a realization that no true studies, such as isotope tracers, have been made on the mechanism of formation. To be sure, Rimpila and Palmer (1935) have made a substantial contribution to the nature of the membrane. These researchers claim that the fat globules may be secreted and the membrane formed even before the milk plasma is completely synthesized. In later work by Mulder (1947), this researcher pictures the fat membrane as arising from the secreting fat globule dragging a thin film of protoplasm from the cell. This assumption could easily explain the presence of such biologically active materials as enzymes, linked intricately together with other materials. From this assumption one could assume that the fat globule served as a clearing mechanism for degenerate cellular tissue. Mulder (1957a) is still convinced of this assumption and expressed doubt that any of the surface layers are formed from the serum itself. However, he is cautious to point out that the secreted fat globule, with its completed envelope, can absorb "a kind of secondary layer of milk serum material."

Richardson and El-Rafey (1948) proposed a hypothesis regarding the origin of the membrane. On the assumption that neutral blood fat

is the main precursor of milk fat, and that blood phospholipids or their complexes with protein take part in the transfer of fat, they postulated that milk fat contains free or linked phospholipid from its early synthesis. Thus, as the globule forms, the surface active phospholipids migrate toward the periphery of the globule with a resulting orientation of the hydrophilic groups of the phospholipid with the hydrophilic side chains of the protein. Primary valence forces act as the main bonding with possible secondary valences. Sommer (1951) visualized that fat globules grow, and in the course of the process phospholipids become associated with the globule, being oriented at the fat-water interface. Fat globules not completely coated with phospholipid continue to merge until a complete monolayer of phospholipid is possible. Recently, Kuiken et al. (1957) reported the presence of a hyalin-fibrin complex in the alveolar bodies of bovine mammary gland. It is conceivable that these bodies ("hyalin" a mucoidal material and "fibrin" a fibrous material) could be sluffed off during the lactation process. It is likewise conceivable that the hyalin-fibrin complex could be strongly hydrophobic and thus have a special affinity for the fat globule surface, and possibly account for the observed presence of the insoluble membrane protein fraction.

Commentary and Objectives

There is little question that "the complexity of the membrane has undoubtedly encouraged some workers to seek areas of more lucrative

data" (Herald, 1956). As a result data on the basic composition of the membrane have not appeared in the literature.

In research today, with the invention of multiple types of electrophoresis units, chromatography of all descriptions, ultracentrifugal apparatus, and countless other tools and methods of isolation, the researcher can no longer rationalize the complexity of a system unless he is simply shy of good, manual labor. There is no question that the insoluble membrane complex is difficult to study, but isolation procedures can partially rectify this situation.

The objectives of this study have been first, an understanding of the basic composition of the fat-globule membrane. In doing so, the lipid portion of the whole membrane has been studied in much detail with particular emphasis being placed on the high-melting glyceride fraction (HMGF), and the chromatographic separation of the entire membrane lipid. The lipid section represents an attempt to elucidate the "fine structure" of the membrane lipid.

Next, since the membrane proteins are considered as minor proteins--on the basis of their low concentrations in bovine milk, they have been compared with the so-called proteose-peptone fractions in an attempt to more fully classify the soluble membrane-protein as well as the proteose-peptone fractions.

Lastly, it was the intent of this study to isolate the true membrane lipoprotein, and to study its unaltered characteristics. Later, the lipoprotein fraction was compared with the soluble membrane-protein of Herald and Brunner (1957) as to similarities and differences. At the conclusion of these studies it was hoped that a logical structure of the milk fat-globule membrane could be presented in order that we know, at least in part, the intricacy with which our Creator has constructed the system.

EXPERIMENTAL PROCEDURE

The milk used throughout this study was from a mixed herd source (Michigan State University creamery) with a fat content of 3.5 - 3.7%, and a total solids of approximately 12.3%. All milk used, except in a specific case to be mentioned, was obtained immediately after delivery. The preparation of protein, lipid and lipoprotein fractions was performed as efficiently as possible, under sanitary conditions, to minimize bacterial and natural enzymatic alterations of the fractions. Since pasteurization was not employed in this study, rapid, efficient handling of the milk was imperative.

Isolation of Soluble and Insoluble Membrane Proteins

The isolation of the soluble and insoluble membrane-proteins was first accomplished by Herald (1956) and later published by Herald and Brunner (1957). The use of organic solvents to remove the membrane lipids was earlier exploited by Palmer and Weise (1933), and Brunner et al. (1953a). Herald's procedure refined the lipid removal method by employing the proper concentration of ethanol in ether to remove the maximum amount of lipid, and to retain the maximum amount of protein nitrogen. Figure 1 shows the schematic isolation procedure for obtaining the soluble and insoluble membrane proteins. The procedure described in Figure 1 varies from Herald's original method in that the raw milk was separated at 40°C. instead of 46°C. The former

temperature was entirely adequate for effective separation of the milk. Secondly, instead of 38°C., a 45°C. temperature was deemed essential to ensure an effective separation of milk fat from the sera. Thirdly, the concentrated membrane was agitated for 30 minutes with the ethanol-ether mixture instead of 15 minutes. This procedure was followed to ensure disruption of the linkage between the lipid and protein portion of the membrane to the maximum extent. No physical alterations of the proteins were observed when this time interval was increased.

Isolation of Lipids and Lipoproteins

Figure 2 represents the schematic isolation procedure for obtaining the high-melting glyceride fraction (hereafter referred to as HMGF) from the milk fat-globule membrane. This fraction was studied earlier in some detail by Jenness and Palmer (1945a) employing crystallization from absolute ethanol. According to Patton and Keeney (1958), acetone serves in the same capacity as ethanol in crystallizing the compounds. The procedure reported here and published by Thompson et al. (1959a) employs acetone initially to remove the bulk of the phospholipids after setting for 30 minutes. A longer duration would undoubtedly precipitate a portion of the HMGF. Following this treatment, the crystallization of the HMGF was performed at room temperature (21-23°C.) employing ethanol as described by Jenness and Palmer (1945a). Subsequent recrystallizations served to further remove impurities as evidenced by increases in the melting

range of the fraction and other properties to be discussed. The initial four washings of the membrane with ethyl ether were designed to remove free fat so that only the tenaciously bound triglycerides would appear in the purified HMGF or its ethanol supernatants. After five crystallizations the HMGF was considered satisfactorily pure for physical and chemical analyses including fractionation by silicic acid chromatography.

The entire lipid of the fat-globule membrane was prepared as shown in the schematic isolation schemes in Figure 3. In contrast to the preparation of the membrane proteins, the temperature of separation was reduced from 40°C. to 35°C. This further reduction in temperature was designed to reduce the rate of enzymatic alteration of lipids as much as possible. Likewise, the temperature of the wash water was reduced from 40°C. to 35°C. Reseparation of the sera was designed to assist in removal of free fat, and was further aided by ethyl ether extraction. It was observed that after lyophilization the lipid-protein complex was disrupted. As a result the lipid was ethyl ether extractable, and ether alone was used for extraction. The final protein-free lipid was stored at -20°C., under nitrogen, to minimize oxidation.

The second scheme (Figure 3, scheme II) was believed to produce a final membrane lipid with a minimum of lipolysis. Thus, fresh, uncooled milk was separated immediately after milking. The scheme also varied in that the sera was ether extracted to remove free fat,

and was not pervaporated before lyophilization, a step omitted to further minimize lipolysis.

Of major interest was the isolation of the true membrane lipoprotein. Since it was well established that producing a suitable density gradient would enhance centrifugal separations, both sodium chloride and sucrose were employed. The first fractionation was designed so that membrane sera possessed a final density of 1.06 g./cm.^{3} after the addition of the appropriate amount of sodium chloride solution. The sodium chloride sera were centrifuged at 25,000 xG for two hours at 22°C., cooled to 4°C. and the fat plug removed. The center cut was decanted and the insoluble pellet discarded. The second scheme shown in Figure 4 represents the schematic isolation procedure used for obtaining membrane lipoproteins employing non-ionizable sucrose as the density gradient. Untreated membrane sera was centrifuged for one hour at 25,000 xG to remove the bulk of the insoluble protein. Sucrose was added to the supernatant to a final concentration of 10%, and the mixture centrifuged 25,000 xG for two hours at 22°C. The plug and pellet resulting from the fractionation were lyophilized, ether extracted, and the lipid prepared for analysis by silicic acid chromatography. The center-cut was analyzed ultracentrifugally before lyophilization so that the lipid-protein complex would not be altered as a result of the dehydration process. Also, a portion of the center-cut was dialyzed, lyophilized, and the lipid extracted. Thus, the sucrose centrifugal fractionation

procedure produced three fractions: (a) the insoluble membrane pellet, (b) the highly dispersed center-cut, and (c) the fat plug at the top of the centrifuge tube.

Isolation of Minor Protein Fractions

This section of the study was designed to determine the similarities and differences between the soluble membrane-protein of Herald and Brunner (1957) and the so-called minor proteins of bovine milk serum. By way of introduction, the "minor proteins" are defined by this author as those serum proteins non-coagulable when heated to 95°C. for 30 minutes. The definition does not mean to imply, however, that heat alteration of the proteins was not a possibility.

Essentially four procedures have been designed to isolate the minor protein fraction. The Rowland fractionation (1938) describes heating milk to 95° C. for 30 minutes, adjusting the pH to 4.6, filtering and precipitating the protein with a final concentration of 10% trichloroacetic acid (TCA). Larson and Rolleri (1955), however, did not employ TCA to procure the Rowland fraction. The isolation procedure for obtaining this fraction is diagrammed in Figure 5. Fresh skimmilk was heated to 95°C. for 30 minutes, diluted, cooled to 20°C., and the pH adjusted to 4.6 with HCl to co-precipitate the heat-coagulated serum proteins with the caseins. After standing overnight in the cold, which assisted in floccing the proteins, the supernatant was decanted and/or centrifuged to remove the residual casein and serum proteins.

After exhaustive dialysis to remove salts and lactose, the fraction was pervaporated, lyophilized, and stored at 4°C. for analysis. This fraction was referred to as proteose-peptone, but was not identical to Rowland's method in that TCA was not employed in the fractionation procedure.

In 1946, Aschaffenburg employed a procedure similar to the above described procedure except that the proteose-peptone was salted out with $(NH_4)_2SO_4$ at 0.5 saturation. Some question exists as to whether Aschaffenburg used acid or rennet whey for procuring the protein fraction from which his experiments were performed. As will be pointed out, the source of whey alters the properties of the protein markedly. Figure 6 outlines the preparative procedure of Aschaffenburg's fraction. This fraction was named sigma-proteose on the basis of its high surface activity, and by virtue of its insolubility in 0.5 saturated $(NH_4)_2SO_4$.

The third procedure employed to obtain a minor protein fraction was that of Weinstein et al. (1951a). This procedure, like those used to obtain proteose-peptone and sigma-proteose, employed heat to coagulate the whey proteins. However, the casein was removed as paracasein after the skimmilk was subjected to the rennin reaction. The coagulum was heated to 50°C. to further expel the whey, and the clear serum heated to 95°C. for 30 minutes. In order to induce flocculation of the heat coagulated whey proteins, the pH was adjusted

to 5.5 after cooling to 20°C., and either allowed to stand overnight, centrifuged at 2,000 r.p.m. for 20 minutes in an International centrifuge (Model V, size 2), or centrifuged 60,000 r.p.m. in a Sharples super-centrifuge. In any case a crystal clear serum resulted. The serum was adjusted to pH 6.7, and the solution 0.5 saturated with $(NH_4)_2SO_4$. The residue was collected, dialyzed salt free against several changes of distilled water, pervaporated and lyophilized. The resulting protein was termed the minor-protein fraction, and its isolation procedure is described in Figure 7.

The fourth procedure for procuring a minor protein fraction was described by Jenness (1959), and appears in Figure 8. Fresh skimmilk (absolutely fat free) was saturated with sodium chloride at 40°C. by the slow addition of the crystalline salt with constant agitation. The mixture was allowed to stand overnight and the bulk of the clear whey decanted. The residue was centrifuged in an International centrifuge for 20-30 minutes (or until reasonably clear), the supernatant decanted, and the residue redispersed in a volume of saturated sodium chloride equal to the original volume. This mixture was centrifuged, the supernatant discarded, and the washing repeated an additional four times. After dialyzing the residue salt free, the mixture was adjusted to pH 4.6, and the casein precipitated. The supernatant, containing serum components 3, 5, and 8 (plus additional protein material), was adjusted to pH 8.0 and passed over an Amberlite IRC-50 H^+ ion

exchange resin to remove lactoperoxidase. The eluate was collected, concentrated, the pH adjusted to 4.6, the supernatant decanted, and the residual protein collected. This fraction was dialyzed, concentrated and lyophilized. The resulting protein fraction was termed specifically by Jenness (1959) as milk component 5, but is referred to in this study as serum or whey component 5. However, this fraction contained reduced amounts of serum components 3 and 8, and additional protein material.

Analytical Methods

Ultracentrifugation. Ultracentrifugal data were obtained from a Spinco Model E analytical centrifuge equipped with a phase plate which was adjusted to obtain maximum focus depending upon the concentration of the solution. The instrument was operated at 20°C. ± 0.01°C. at speeds of 52,640 r.p.m. (219,960 xG) or 59,780 r.p.m. (259,700 xG) utilizing the AN-D rotor with an AN cell of 6 mm. centerpiece thickness. Acceleration times for the above two speeds were 8 and 10 minutes, respectively, as controlled by the Variac. Metallographic plates were exposed for 8 seconds and developed for 5-8 minutes.

Proteins were carried in buffers as indicated. Concentrations of at least 1% were desired, but in many cases this was impossible due to the nature of the solubility of the proteins.

Calculations were made from the following equation:

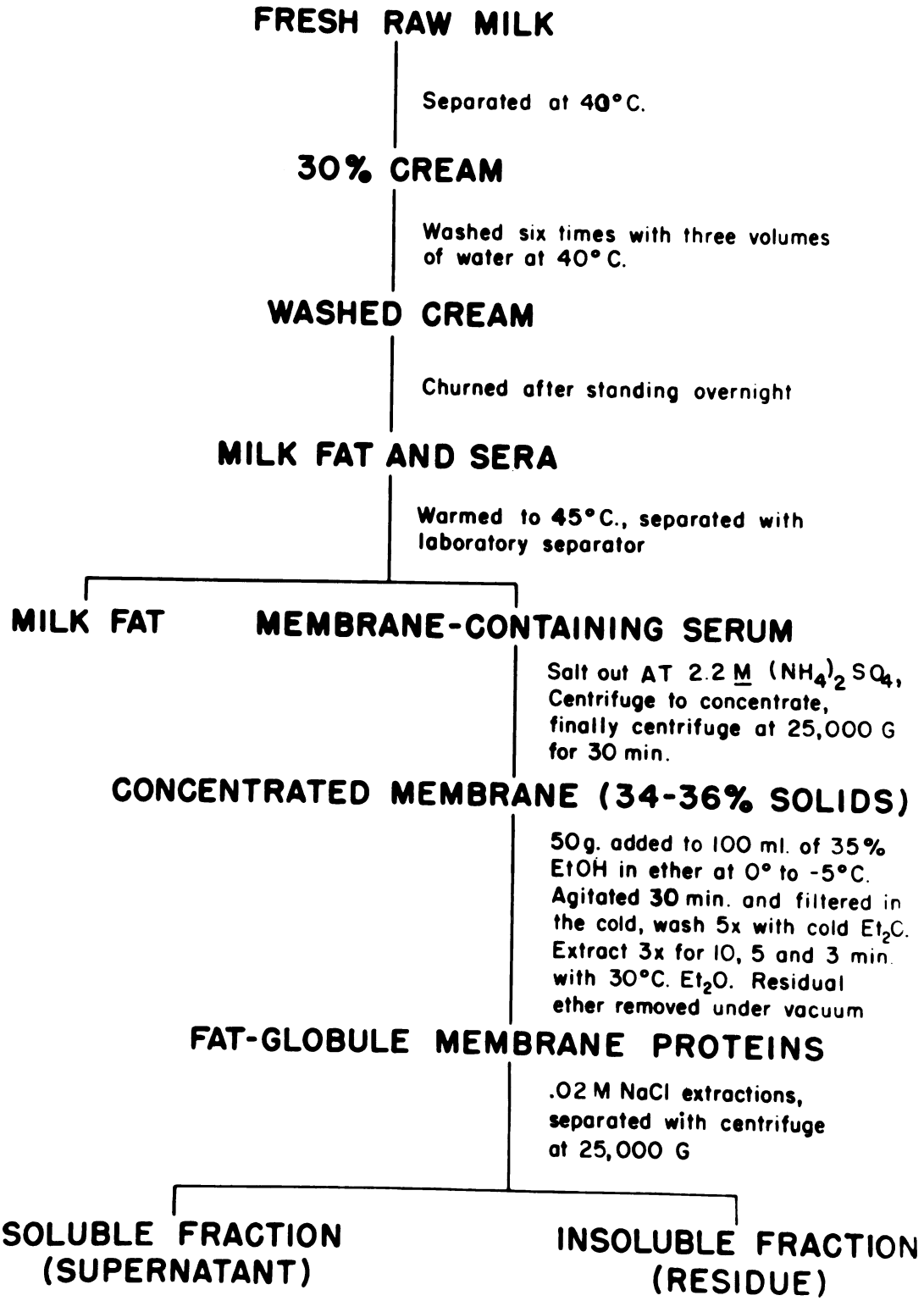


Figure 1. Schematic isolation procedure for obtaining the insoluble and soluble membrane proteins according to Herald (1956).

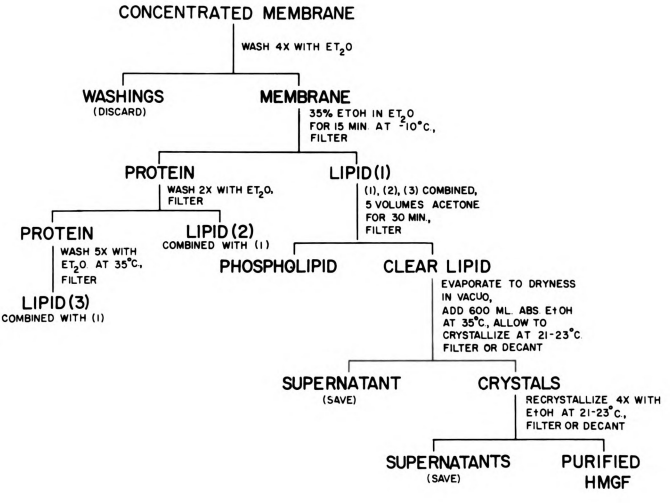
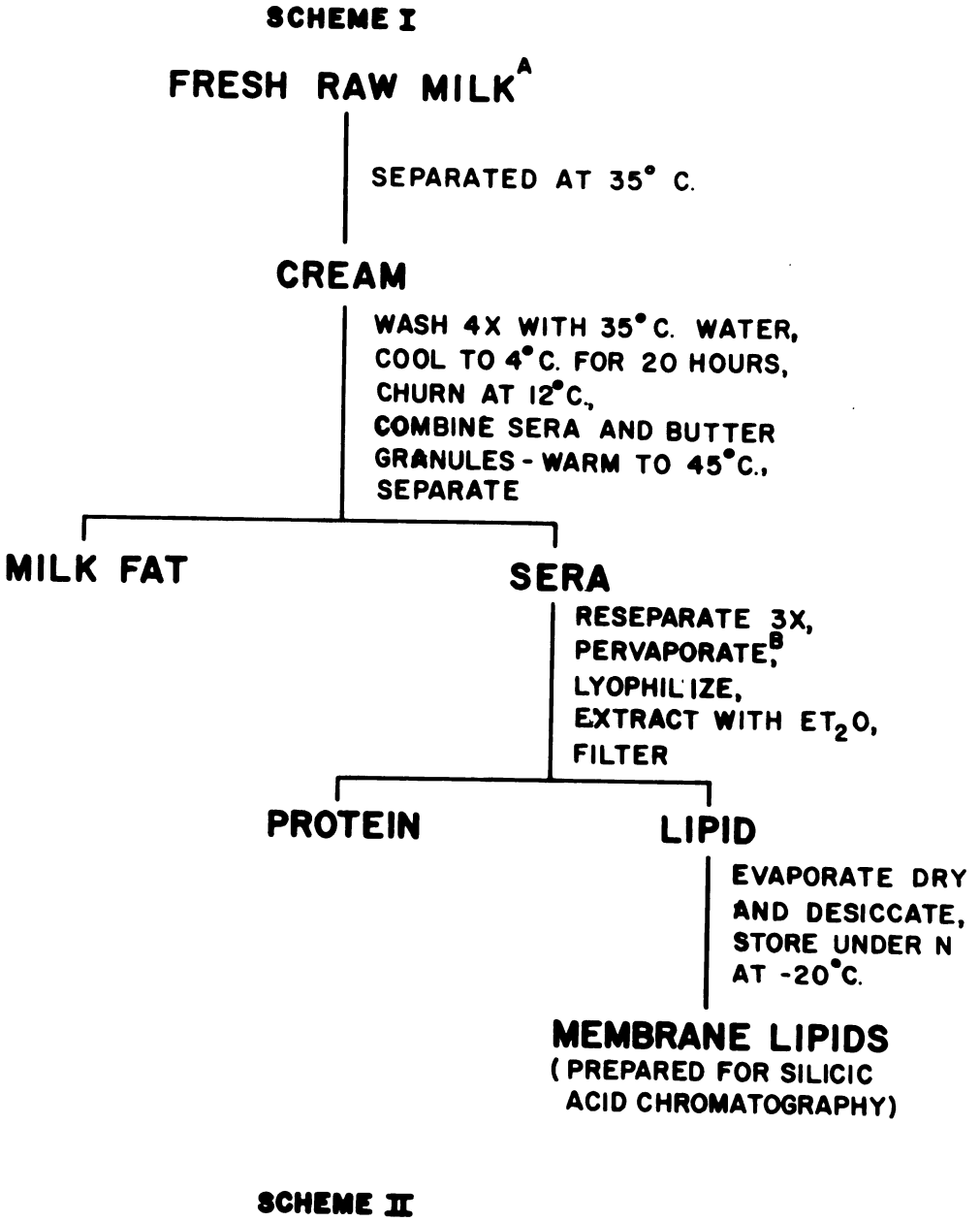


Figure 2. Schematic isolation procedure for obtaining the high-melting glyceride fraction from the milk fat-globule membrane.



SCHEME II

SCHEME II VARIES FROM SCHEME I AS FOLLOWS:

A. WARM, FRESH, WHOLE MILK WAS SEPARATED SHORTLY AFTER MILKING WITHOUT BEING COOLED.

B. THE SERA WAS WASHED 2X WITH ET_2O TO REMOVE FREE FAT, AND WAS NOT PERVAPORATED BEFORE LYOPHILIZATION.

Figure 3. Schematic isolation procedure for obtaining complete membrane lipids for fractionation by silicic acid chromatography.

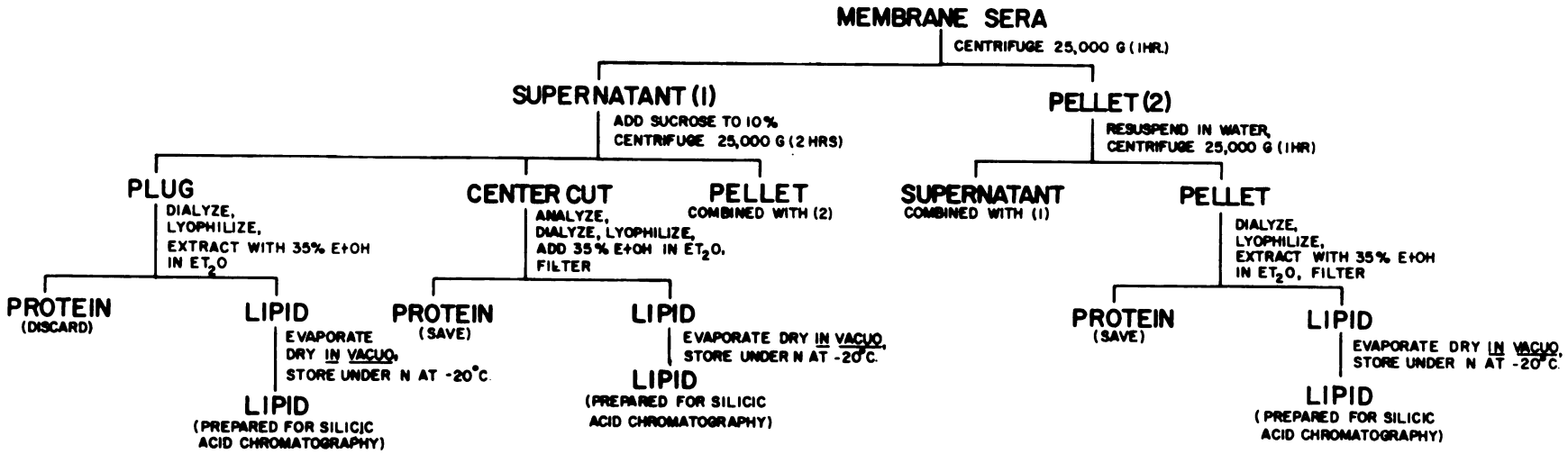


Figure 4. Schematic isolation procedure for centrifugally separating fat-globule membrane sera into its component protein fractions using sucrose for the density gradient.

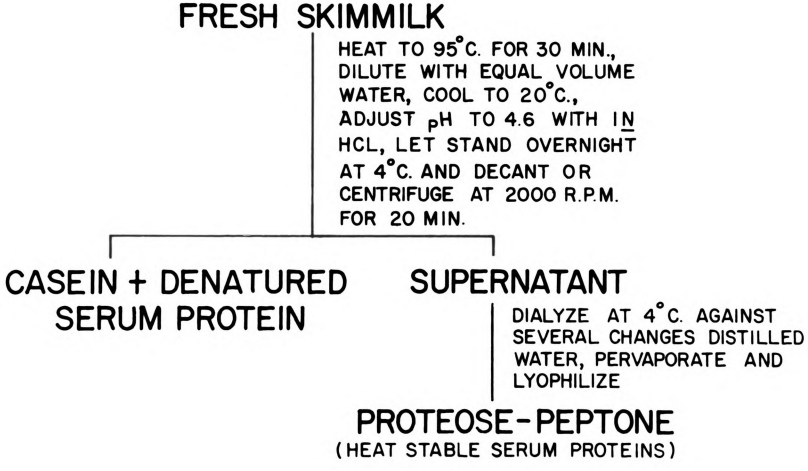


Figure 5. Schematic isolation procedure for obtaining the proteose-peptone fraction from skimmilk.

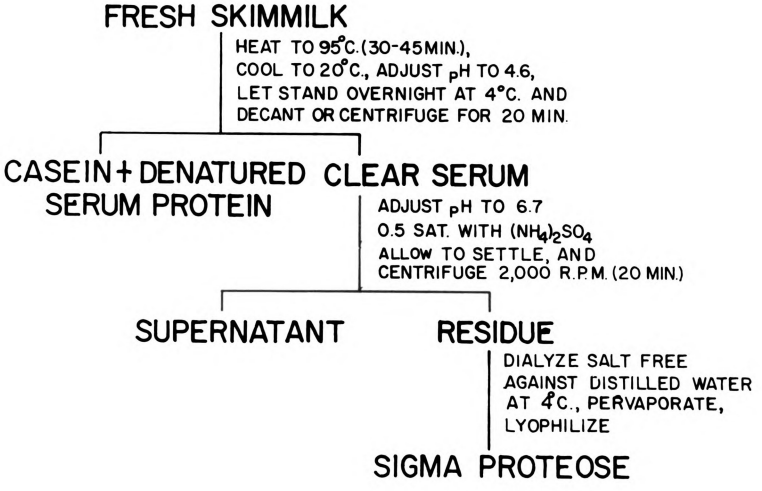


Figure 6. Schematic isolation procedure for obtaining the sigma-proteose fraction from skimmilk according to Aschaffenburg (1946).

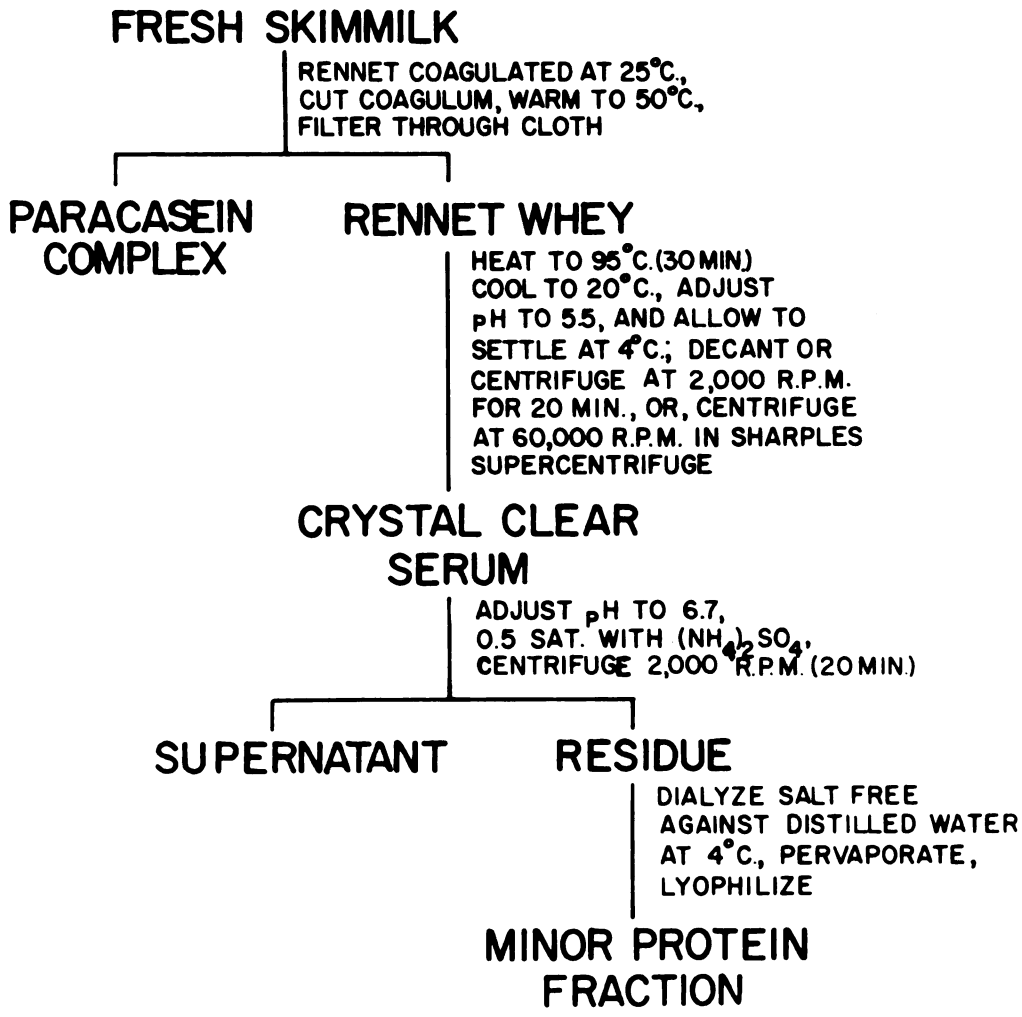


Figure 7. Schematic isolation procedure for obtaining the minor protein fraction of Weinstein et al. (1951a) from skimmilk.

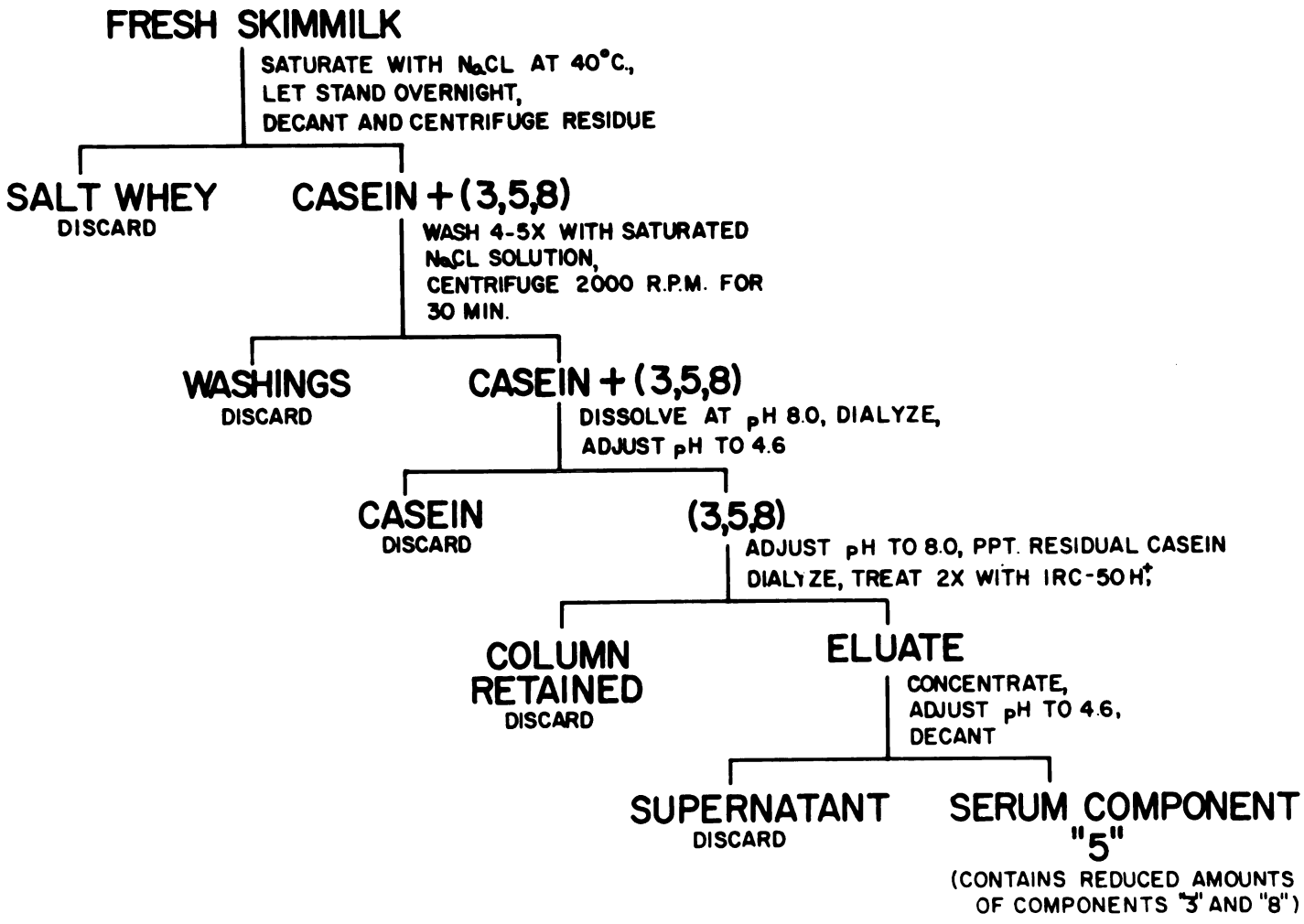


Figure 8. Schematic isolation procedure for obtaining serum component 5 from skimmilk according to Jenness (1959).

$$S_{20}\,(\text{uncorrected})^{*} = \frac{\frac{d}{m}}{39.5\ t\ r.p.s.^{2}\ r}$$

where:

d = distance migrated in cm.

m = magnification factor for the instrument - 2.1

$39.5 = 4\pi^{2}$

t = time in seconds

r = distance from center of rotor to a position at the center of the component, which it would be if an exposure were made halfway between the pair of peaks used for a calculation. 5.72 cm. was the distance between the center of the rotor and the air reference line of the cell when operated at 59,780 r.p.m. S_{20} values are represented by a measurement and calculations of at least three frames.

Electrophoresis. Free-boundary electrophoresis was carried out in a Perkin-Elmer Model 38 electrophoresis unit equipped with a cylindrical lens. A temperature of 1°C. was maintained in earlier studies by means of crushed ice; whereas in later studies water at 1°C. was constantly circulated throughout the water bath. Modified Tiselius cells, either 2 ml. or 6 ml., were employed. An Industrial Instruments Conductivity bridge (Model RC-16) with a 2 ml. cell having a constant of 0.8491 was employed to determine the resistance of buffer and protein solutions at 0-1°C.

*Denotes uncorrected for viscosity, and extrapolation to zero concentration.

Ideally, 1% and 0.5% protein solutions were desirable for the 2 ml. and 6 ml. cells, respectively.

Electrophoretic mobilities were calculated by using the following equation:

$$\mu(cm.^2\ volt^{-1}\ sec.^{-1}) = \frac{d\,a\,k}{t\,i\,r\,m}$$

where:

d = distance migrated from initial boundary

a = cross-sectional area of cell

k = conductivity constant = 0.8491

t = time in seconds

i = amperes

r = resistance of buffer in ohms

m = magnification factor of the optical system - 1.1

Field strength ($V.\ cm.^{-1}$) was calculated by use of the equation:

$$F = \frac{i}{ak}$$

where:

F = field strength

i = amperes

a = cross-sectional area of cell

k = specific conductivity of the buffer protein solution

Peak areas were determined by preparing 5-fold magnifications of the photographic print, drawing a perpendicular line from the lowest

point between peaks, cutting out the area, and weighing the component parts. Electrophoretic mobilities are reported on both ascending and descending channels with selection of mobilities left up to the reader's judgment. Comparative interpretation by the author has been made employing either channel depending upon the literature cited.

Ultraviolet, visible and near infrared. These regions of the spectrum were studied with a Beckman DK-2 ratio recording spectrophotometer within specific wave lengths which appear in the results. All data are plotted as absorbance (0-1) with spectrum scanning times of 1-2 minutes for ultraviolet and visible, and 5-minute for near infrared.

Concentrations were chosen so as to obtain a maximum absorbancy for the sample of 0.50 - 0.75 units, the absorbancy being controlled by dilutions or reduction of the cell path from 1.0 cm. to 0.1 cm.

Infrared. Infrared studies were performed with a Beckman IR-5 double-beam spectrophotometer with an operational range of 2-14 μ. All lipid samples, carried in CCl_4 at a concentration of 5%, were analyzed in a liquid cell of 0.2 ml. capacity and a path length of 0.002 cm. CCl_4 served satisfactorily to qualitatively discern spectral qualities of various lipids although CS_2 is equally satisfactory.

Silicic acid chromatography. This portion of the study employed silicic acid chromatography to separate lipids of the fat-globule membrane and other fractions described. The entire procedure is aptly described by Hirsch and Ahrens (1958). However, a few important aspects of the procedure are mentioned here. The column was packed with 18 grams of

minus 325 mesh silicic acid as supplied by Bio-rad (Richmond, California). The scheme of gradient elution was followed using ethyl ether, hexane (b. p., 60-70°C.), and absolute methanol, all of which were glass distilled before use. Flow characteristics of the column gave an elution rate of approximately one ml. per minute without employing nitrogen pressure. Preliminary trials showed that the omission of nitrogen pressure did not affect the degree of resolution of eluted components, but did affect the position of elution. Gjone et al. (1959) stated that the omission of nitrogen pressure when eluting phospholipids enhanced rather than hindered the degree of resolution. The column was charged with quantities of lipids in excess of 300 mg. in all cases. Overloading was noted when the lipid charge exceeded 400 mg., but this was not a serious defect in the operation.

To determine the amount of the various lipids eluted from the column, clean test tubes were accurately weighed on an analytical balance to ± 0.1 mg. under constant humidity conditions. After collection of the lipids eluted, the solvent was evaporated in an air circulated oven maintained at temperatures slightly below the boiling points of the solvents. In this manner, violent eruptions of solvents were avoided. The tubes were then equilibrated for 20 hours under the same conditions as the original weighing, and the weight recorded. Ten blank tubes were maintained throughout an entire elution run. These tubes were weighed and at no time did their average weight vary more than ± 0.1 mg.

It was realized that drying in a non-inert atmosphere and in the presence of heat could easily induce lipid oxidation. However, since gross infrared spectral properties were not altered by this procedure, this factor was considered not to markedly affect the overall value of the results.

To insure that elution of unknown lipids was uniform, a standard known series of lipid classes was run on the silicic acid column. The standard lipid classes were obtained commercially.

Refractive indices. Refractive indices were performed at 60°C. in a Bausch and Lomb refractometer using undiluted lipids.

Partition paper chromatography. Twenty-five ml. of a solution containing 125 mg. of protein were hydrolyzed at 100°C. for 3 hours with 2 N HCl. Several trials showed these conditions to be optimum for liberation of carbohydrates from the protein. The hydrolysates were dialyzed against 3 changes of distilled water which were combined, evaporated to dryness, and extracted with pyridine to remove salts (Block et al., 1958). The residue obtained after vacuum removal of the pyridine, was dissolved in a minimum of 10% isopropanol, and spotted on Whatman No. 1 filter paper. A descending chromatogram was run for 32 hours at 21°C. using a solvent mixture made up of amyl alcohol, pyridine, and water (4:3:2). The chromatogram was dried at 70°C. for 15 minutes, and developed at 40°C. in a moist atmosphere with 2% triphenyltetrazolium chloride in an equal volume of 1 N NaOH

(Block et al., 1958). This developing agent proved superior to either ammoniacal $AgNO_3$ or aniline oxalate as far as this study was concerned. Known carbohydrates were spotted similarly and served as standards in the identification of spots from the protein samples. The complete procedure appeared in a publication by Thompson and Brunner (1959c) and reported a study of the carbohydrates of the soluble membrane-protein of Herald and Brunner (1957), the minor-protein fraction of Weinstein et al. (1951a), and the proteose-peptone fraction of Rowland (1938).

Intact carbohydrate analyses. Hexose, hexosamine and neuraminic acid were analyzed by the procedures outlined by Glick (1955). Galactose, and galactosamine were chemically pure standards whereas neuraminic acid was obtained using orosomucoid from human blood serum as a secondary standard (11. 2% neuraminic acid). Protein samples were of the magnitude of 5 mg. for each study performed.

Ashing of protein. One gram samples of protein were ashed at 600° C. after the addition of 1 ml. of 50% $MgNO_3$ (to hold the phosphorous) for a period of 20 hours. If charred particles were present after this period of time, the ash was re-wetted and re-ashed for an additional 12 hours.

Iodine values, saponification equivalents, and melting ranges. Iodine values, and saponification equivalents were obtained according to standard procedures (A. O. A. C., 1955). Melting ranges were performed on lipid samples in capillary tubes in a rising temperature water bath.

Methanolysis and vapor phase chromatography. The high-melting glyceride fraction and butteroil were prepared for gas chromatographic analysis by methanolysis in Skellysolve A according to the method described by Smith and Jack (1954a). Excess solvent was removed in vacuo. The resulting fatty acid methyl-ester mixtures were resolved on a Burrell gas chromatographic unit. Identification of the unknown chromatographic peaks was achieved by comparing their elution characteristics with those from similarly prepared chromatograms of a known fatty acid mixture, and by employing the known mixture as an internal standard. The chromatographic column was packed with 18.6 g. of 20% (w/w) Reoplex 400 on 60-80 mesh Celite 545, and was operated at a temperature of 224°C. The detector was maintained at 238°C. while the preheater was operated at 263°C. Helium was employed as the carrier gas with an outflow rate of approximately 50 cc./38.9 sec. at C_{18} for all chromatographic runs. The complete procedure appeared in a publication by Thompson et al. (1959a).

Nitrogen. Nitrogen analyses were made in duplicate on 100 mg. protein samples by the Kjeldahl method or on appropriate amounts of aliquot samples in solution. Twenty ml. of concentrated H_2SO_4 were used to digest the samples until clear and 15 minutes longer. The Kjeldahl flasks were cooled, neutralized with 50% NaOH, distilled into a boric acid solution, and titrated with 0.02 N HCl.

Phosphorous. Phosphorous analyses were performed on the ashed proteins by extracting the ash with dilute HCl and developing the color with ammonium molybdate according to the procedure of Fiske and Subbarow (1925).

Fat and total solids. Fat and total solids were determined by the gravimetric method of Mojonnier and Troy (1925). However, when lipid analyses were determined the addition of ammonium hydroxide was approximately doubled. This addition served to more fully degrade the protein.

Solubilization of the insoluble membrane protein. Brunner and Herald (1957) previously reported several methods for solubilizing the pseudokeratin-like protein fraction of the milk fat-globule membrane. The protein fraction was solubilized, in this study, by three methods: (a) adjustment of the pH to 11.8 with 1 N NaOH, (b) the addition of a solution of 3% peracetic acid, and (c) the addition of 2' thiodiethanol until the solution cleared.

Figure 9. Photograph of equipment employed in silicic acid chromatography of membrane and butteroil fractions.

RESULTS AND DISCUSSION

Since a considerable amount of divergent data are contained in this thesis, the author felt justified in dividing the thesis into three sections, each including experimental results and discussion. The first section is dedicated to a discussion of the high-melting glyceride fraction (HMGF) and to the chromatographic analyses of the entire membrane lipid material and butteroil. The second section served in the twofold capacity of comparing the membrane proteins obtained in this study with those reported in the literature, and to compare the minor protein fractions of bovine milk with the soluble membrane-protein. The third and last section has been designed to discuss the true membrane lipoprotein. This section includes the analytical studies of the soluble and insoluble membrane-proteins for comparative purposes. At the conclusion of the three sections, a summary and conclusion of the data is given from which the author intends to depict a logical structure of the milk fat-globule membrane.

High-melting Glyceride Fraction (HMGF)

Although Jenness and Palmer (1945a) studied the physical characteristics of the HMGFraction in some detail, the exact nature of the fatty acid residues plus other valuable data was not presented. Nevertheless, the Palmer group contributed significantly to the recognition of the importance of this fraction.

As was previously described, the HMGFraction was prepared by crystallization from ethanol after a pretreatment with acetone to remove the bulk of the phospholipids. After each crystallization, a sample of the ethanol supernatant was run in the ultraviolet region to determine the extent of removal of ethanol soluble materials which, if present, would serve to mask the true spectral characteristics of the HMGF. These spectra appear in Figure 10. Following the third extraction, the spectrum of the ethanol supernatant began to resemble that of the HMGF shown in Figure 10, B. The first and second extractions, which were yellow in color, showed spectra quite dissimilar to those of the subsequent extractions. Apparently this was due to the presence of absorbing materials such as sterols, beta carotene, or phospholipids. These data indicate, then, that four extractions with absolute ethanol were required to free the membrane HMGF of its major impurities. The ethanol extracts were combined, evaporated to dryness in vacuo, and retained for further study.

The iodine value, saponification equivalent, and melting range of the HMGFractions from both the fat-globule membrane and butteroil were compared with similar values from the glyceride fractions by Jenness and Palmer (1945a) and Patton and Keeney (1958), Table 1. A difference exists between the two reported iodine values of the two HMGFractions reported here and the HMGF obtained by acetone fractionation from milk fat by Patton and Keeney (1958). This difference

might be attributed to the method of preparation; that is, ethanol extractions as opposed to acetone extractions.

The ultraviolet spectra of the HMGFractions from the fat-membrane and butteroil are compared in Figure 11. These fractions show identical spectral characteristics with five absorption maxima at 315, 301, 279, 267 and 256 mμ, respectively. Smith and Jack (1954b), reporting on the unsaturated fatty acids of milk fat, presented almost identical spectral characteristics, showing absorption peaks at approximately 313, 300, 280, 268, and 233 mμ, but not at 256 mμ. These spectral qualities characterize the presence of unsaturation in the glycerides, but since alkali isomerization was not employed, the data cannot be interpreted to ascribe the type of conjugation.

In recent years, the near-infrared region of the spectrum (2.7 - 3.0μ) has been useful for the study of functional groups, especially in the identification of hydroperoxides (Slover and Dugan, 1958). However, the vibrational spectra of compounds in this region are exceeding difficult to interpret. Figure 12 shows the near-infrared spectra for the HMGF and totally saturated tristearin. An examination of the spectra reveals marked dissimilarity between these compounds in the region of 2.7 - 3.0 μ, a dissimilarity obviously arising from the nature of the molecule; i.e., unsaturation within the HMGF.

The infrared spectra (2-14 μ), which reflects both vibration and rotation of molecules, is given in Figure 13 for the HMGFractions and

tristearin. Although this region is exceeding useful for "fingerprinting" functional groups, some researchers are prone to oversimplify their interpretations. In the case of lipids, for example, the spectra of the fatty acids correspond closely to the related spectra of triglycerides differing primarily in that a carboxyl OH has replaced the ester carbonyl between the fatty acid residue and glycerol. Figure 13 shows that the infrared spectra of the HMGFractions were truly triglyceride, and the recorded spectra corresponded closely to those reported by Patton and Keeney (1958) who used CS_2 as the lipid solvent rather than CCl_4 as employed here.

One of the most interesting parts of this study proved to be the identification of the fatty acid residues of the HMGFractions which certainly contributed to a better knowledge of the physical properties of the compound. Gas chromatograms of the methyl esters of the fatty acids of butteroil and the membrane HMGF from the same milk, and a mixture of known fatty acids (C_{10} to C_{18}, and $C_{18}^{1=}$ to $C_{18}^{3=}$) are shown in Figure 14. These chromatograms were analyzed and compared with similar data reported by Patton and Keeney (1958), Table 2. The first obvious feature of the gas chromatograms is the remarkable resolution afforded by the Burrell, thermal conductivity cell. Butteroil, for example, is highly resolved to C_6 carbon fragments. The membrane HMGF reported here contained approximately 9 $\underline{M}$% more C_{16} and 9 $\underline{M}$% less C_{18} saturated acids, and 2.0 $\underline{M}$% more C_{18} unsaturated acids than the glyceride reported by Patton and Keeney (1958). One would

suspect that this fraction would possess a lower melting range than those of Patton and Keeney, but they failed to report these data.

Specifically, the C_{18} unsaturated acids have been identified as $C_{18}^{1=}$ - 5.6 M%, $C_{18}^{2=}$ - 0.4 M% and $C_{18}^{3=}$ - 0.2 M%. No analyses were made for $C_{18}^{4=}$. Unassigned peaks accounted for 5.8 M% in the HMGF as compared to a remarkably close value of 5.9 M% reported by Patton and Keeney (1958). Butteroil itself contained 10.5 M% unassigned peak areas which could be attributed to odd-numbered fatty acids or branched fatty acids. The gas phase chromatogram of butteroil was presented to establish the nature of the original milk fat, and the results correspond closely to those in a recent publication by Patton et al. (1960).

The gas chromatographic results certainly reveal many important features concerning the HMGF. Namely, a total mole % of 6.2 for the unsaturated fatty acid residues accounted for the low iodine value of 4.8 - 5.0. Secondly, the predominance of C_{14}, C_{16}, and C_{18} was the determining factor for the high melting range of 50.0 - 52.5°C., the relatively low saponification equivalent of 200-202 (which is inversely proportional to the molecular weight), and the refractive index of 1.4453 - 1.4455 which is proportional to the fatty acid chain length and degree of unsaturation. The data thus far established that the HMGF constituted a large part (44.48%) of the bound lipid of the fat membrane. In this area, it was interesting to note that all combined ethanol extracts, obtained after crystallization of the HMGF,

contained very few fatty acids. This discovery prompted Thompson et al. (1959a) to propose that the HMGF constituted the major portion of the membrane triglyceride. This find also suggested that the crystallization of the HMGF was remarkably complete.

The relative homogeneity of the HMGF prompted the author to study the fraction by silicic acid chromatography to determine if the fraction could be separated into its component triglyceride fractions. Employing the procedure of Hirsch and Ahrens (1958), which will be discussed more fully in the following lipid study, the HMGF was chromatographed. Figures 15 and 16 show the results of this study. The fractions eluted sharply, as shown in Figure 15, when 10% ethyl ether was used in the top reservoir for gradient elution. However, when the ethyl ether concentration was reduced to 5%, Figure 16, much heterogeneity became apparent. Keeney (1960) reported that the di-stearic acid containing triglyceride could be obtained by silicic acid chromatography of the HMGF. An analysis of this fraction is now under consideration in this laboratory.

Table 1

Properties of the HMGF isolated from the fat-globule membrane and from milk fat

Analysis	From the fat membrane (this study)	From milk fat (this study)	From the fat membrane (29)	From milk fat (49)
Iodine value	5.0	4.8-4.9	5.0-7.1	2.0
Saponification equivalent	201-202	200-202	198.8-204	--[c]
Melting range	50-51.5°C.	50-52.5°C.	52-53°C.	--[c]
Refractive index at 60° C.	1.4455	1.4453	--	--[c]
Yield	44.48[a]	4.88%[b]		

[a]Percentage of bound lipid in membrane

[b]Percentage of original butteroil

[c]Reported by Patton and Keeney (1958) to be similar to value reported by Jenness and Palmer (1945a)

Table 2

A comparison of the fatty acid composition of the fat-globule membrane HMGF, butteroil from the same preparation, and a HMGF isolated from milk fat

Fatty acid	HMGF from the fat membrane[a]	Butteroil	HMGF from milk fat (49)	
			A-1	B-1
	Mole %			
C_{10}	0.1	3.9	0.3	0.7
C_{12}	0.9	4.3	1.4	1.6
C_{14}	11.0	12.6	12.1	13.6
C_{16}	59.6	25.5	50.5	51.6
C_{18} sat.	16.5	11.3	25.5	30.4[c]
C_{18} unsat.	6.1	28.3	4.1[b]	--
$C_{18}^{1=}$	5.6	25.8		
$C_{18}^{2=}$	0.4	.3		
$C_{18}^{3=}$	0.2	2.2		
Unassigned peak areas	5.8	10.5	5.9	--

[a] Identical gas chromatograms were obtained for both the membrane and butteroil HMGFractions.

[b] Total C_{18} unsaturated

[c] Includes C_{18} unsaturated

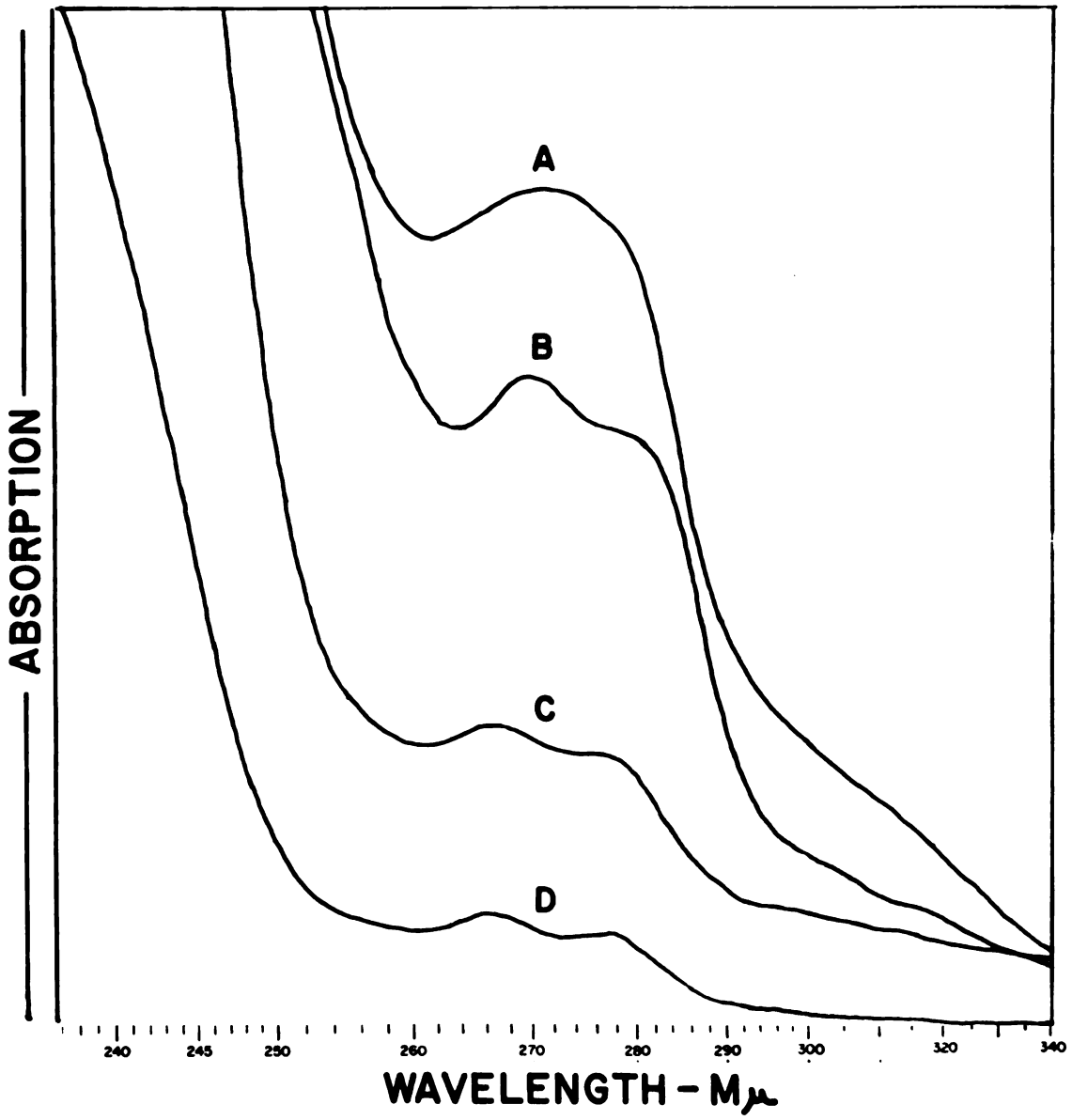


Figure 10. Ultraviolet spectra of ethanol extracts of the high-melting glyceride. Curve A, first extract, diluted 1:2, 0.1 cm. path. Curve B, second extract, diluted 1:1, 0.1 cm. path. Curve C, third extract, undiluted, 0.1 cm. path. Curve D, fourth extract, undiluted, 1.0 cm. path.

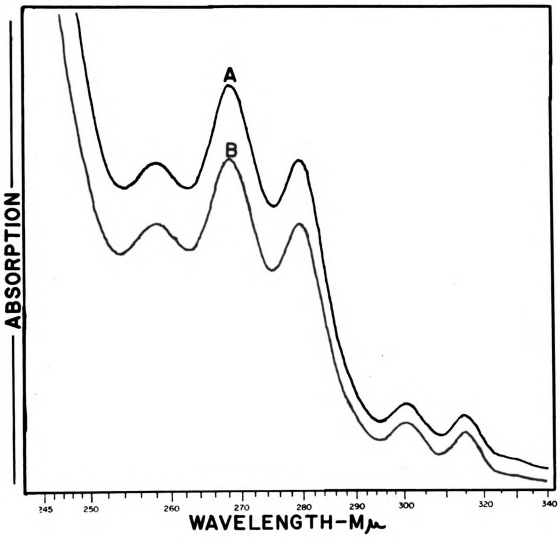


Figure 11. Ultraviolet absorption spectra of the high-melting glyceride fractions obtained from (A) butteroil and (B) the fat-globule membrane.

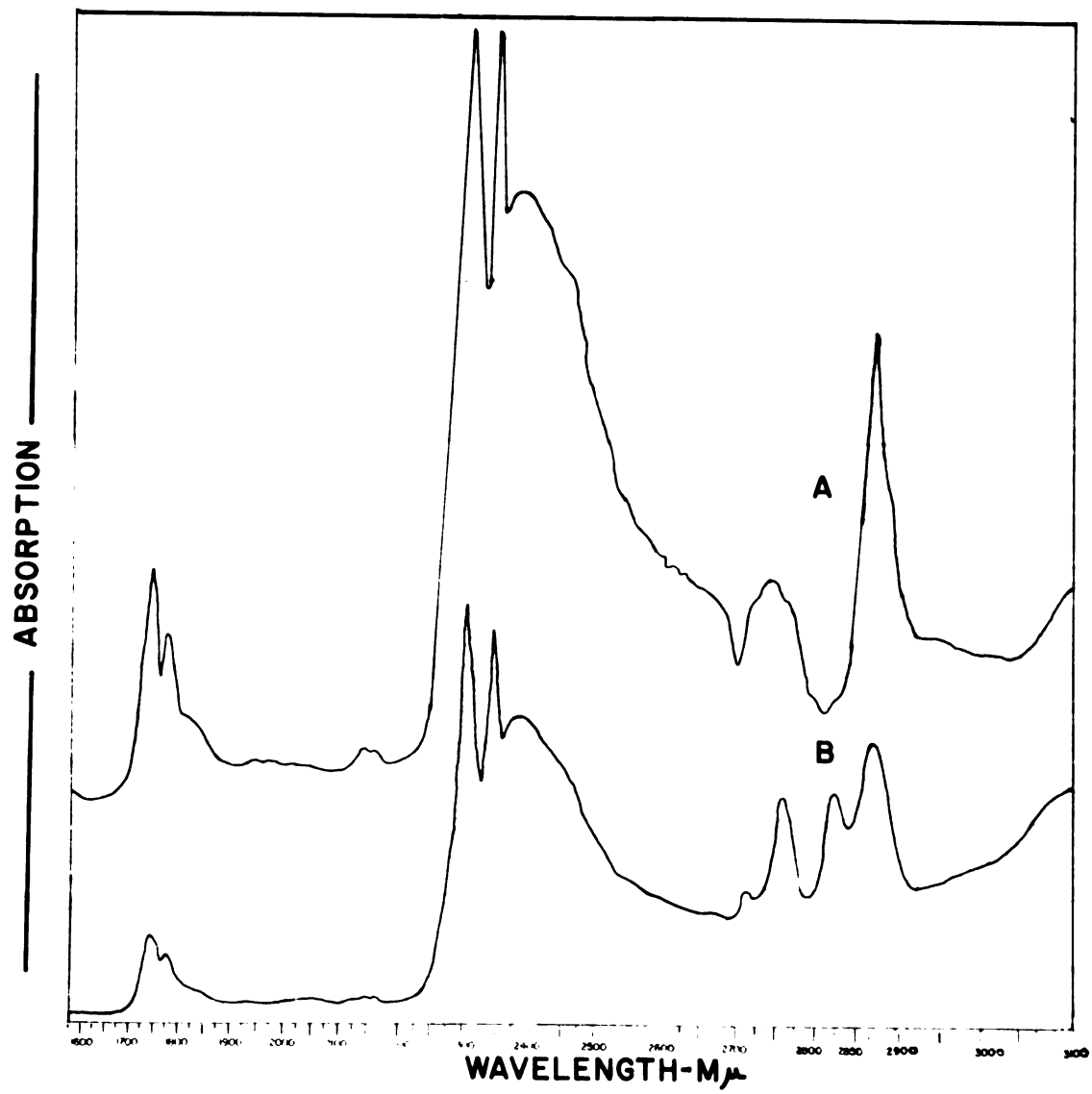


Figure 12. Near-infrared (photoelectric) absorption spectra of (A) tristearin and (B) the high-melting triglyceride in CCl_4. Concentrations approximate 5%.

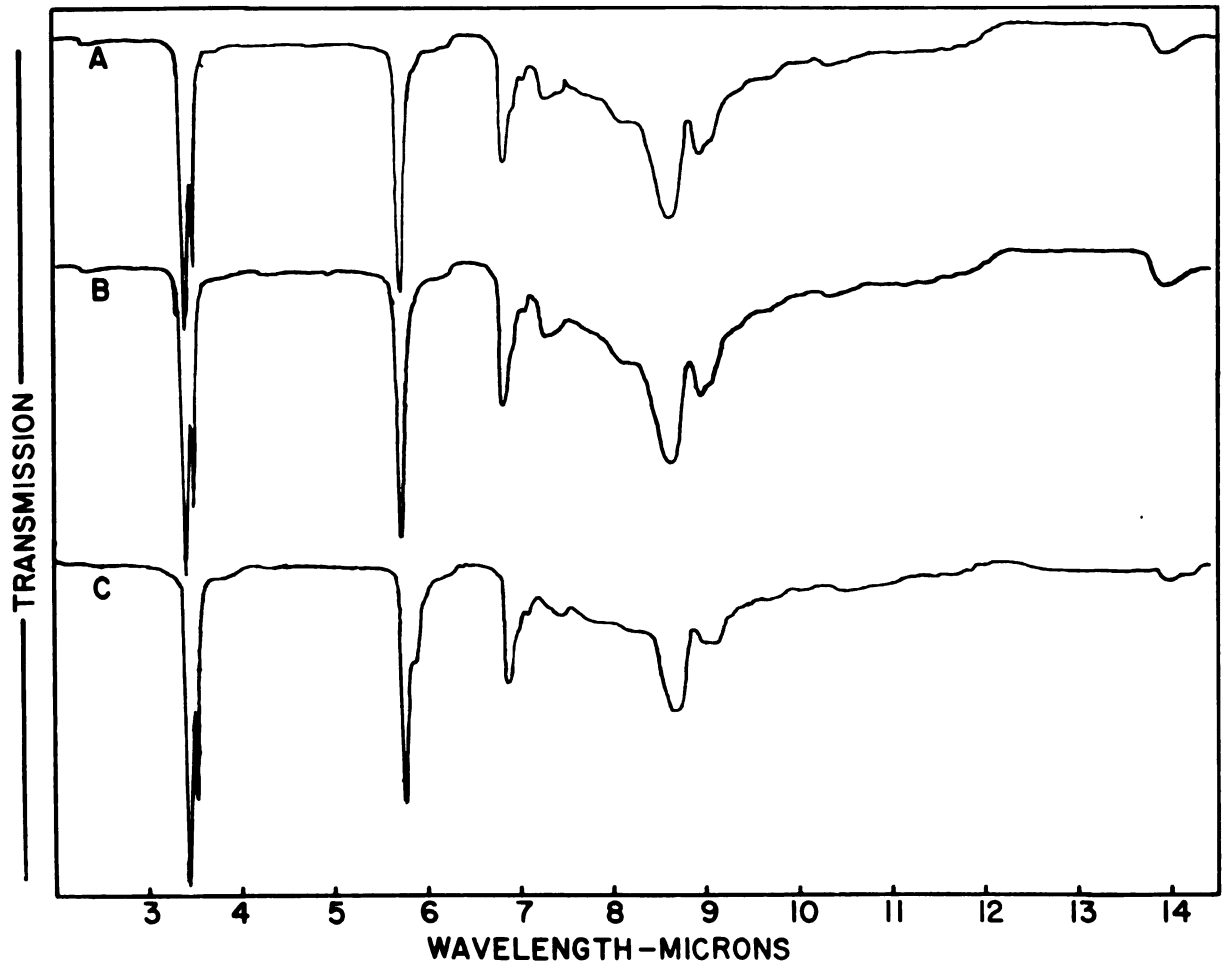


Figure 13. Infrared spectra of (A) HMGF from butteroil, (B) HMGF from the fat-globule membrane, and (C) tristearin. Concentration was approximately 5%.

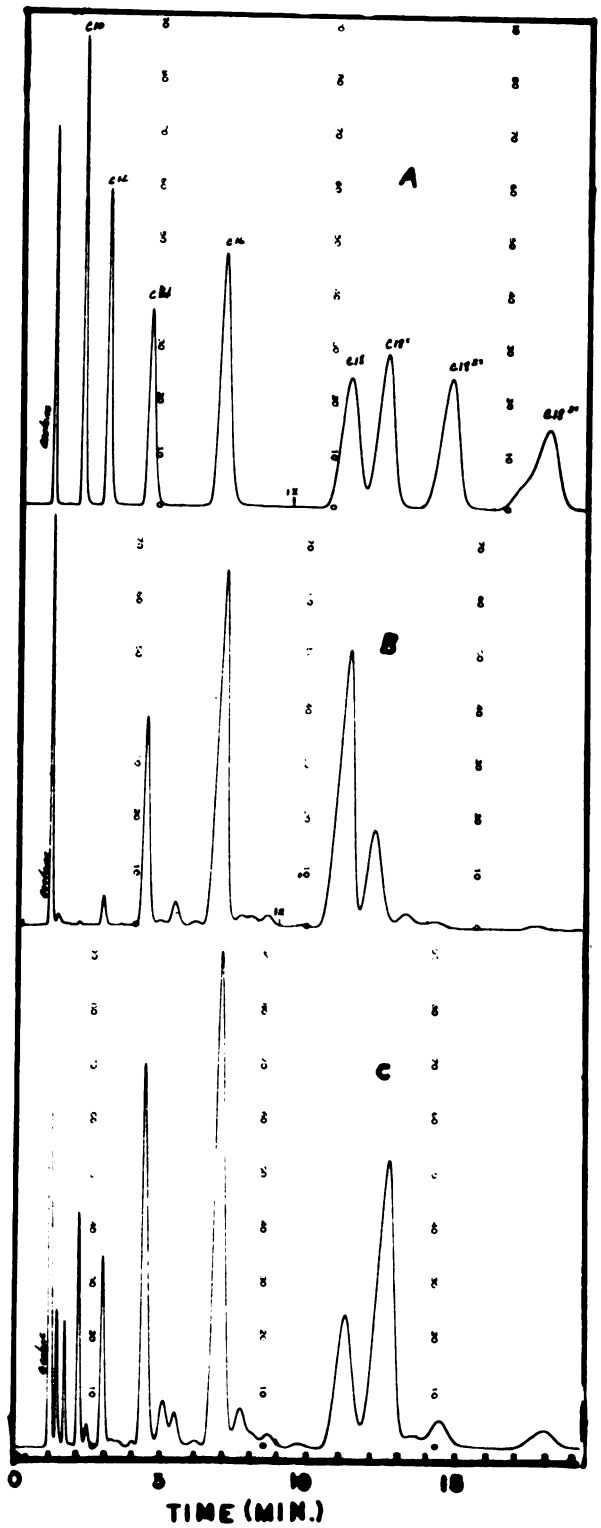


Figure 14. Gas chromatograms of the fatty acid methyl esters of (A) a known mixture of fatty acids, (B) the fat-globule membrane HMGF, and (C) butteroil. 1x denotes time of a twofold increase in detector sensitivity.

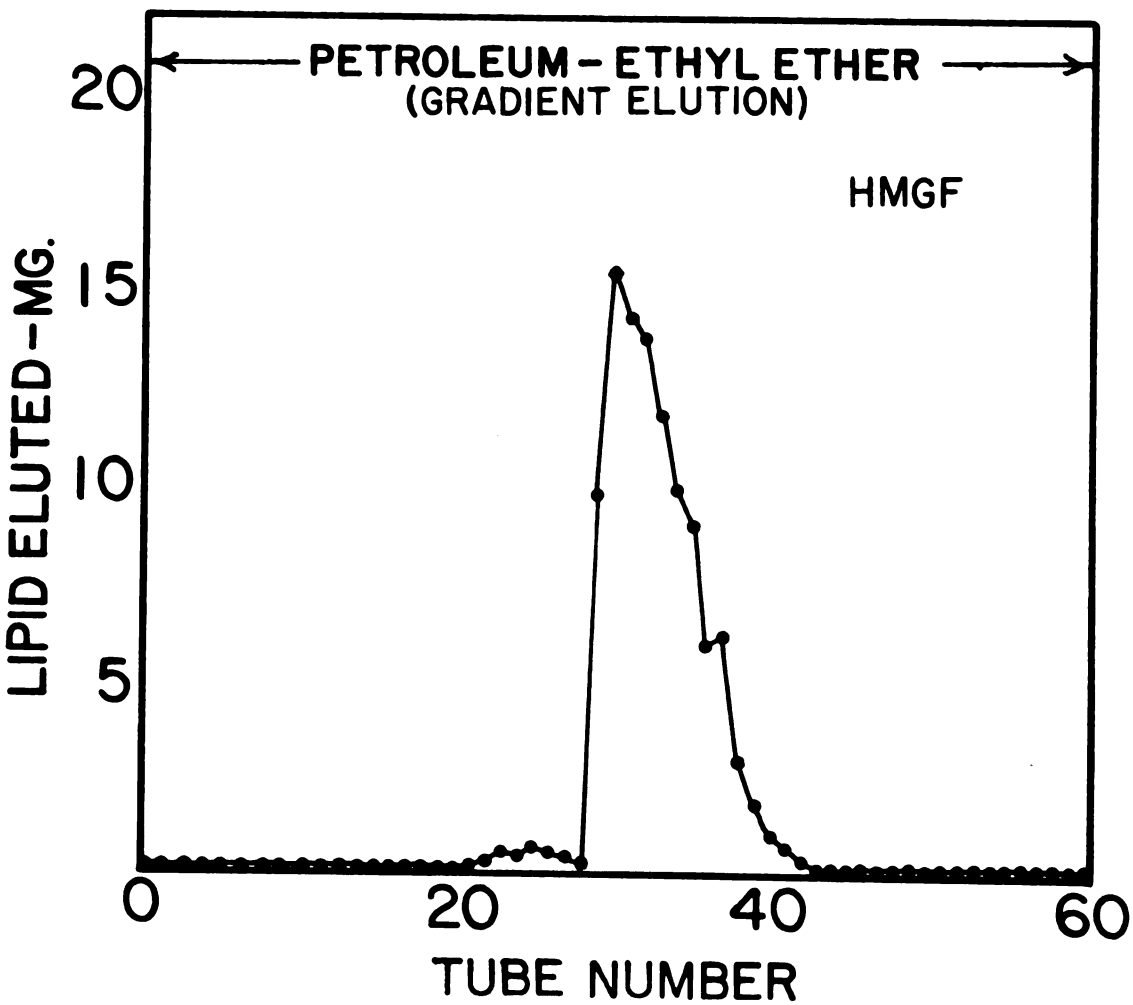


Figure 15. The separation of the high-melting glyceride fraction by elution from an 18 g. column of silicic acid. Ten percent ethyl ether was employed in the top reservoir with a lipid charge of 108.3 mg.

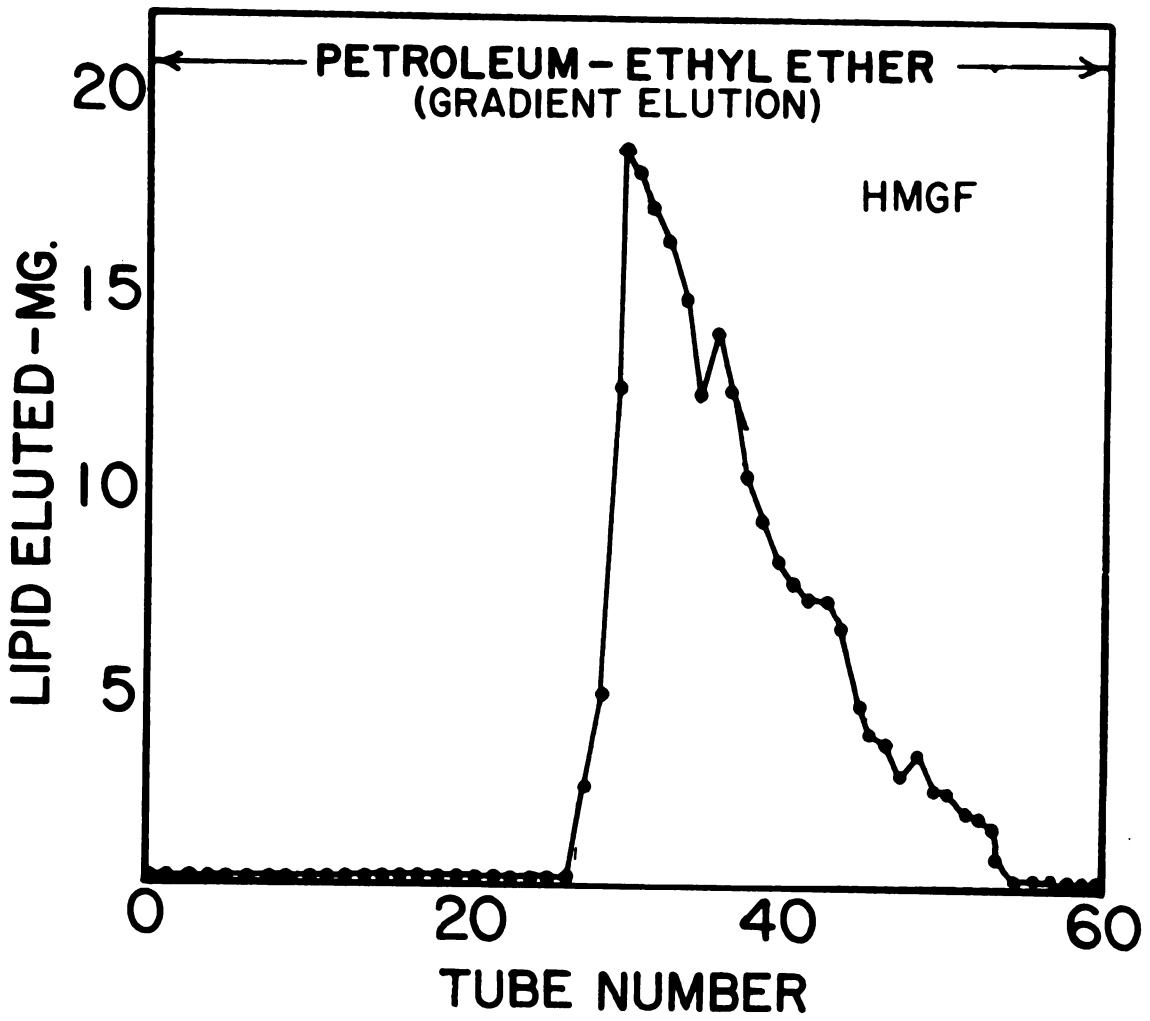


Figure 16. The separation of the high-melting glyceride fraction by elution from an 18 g. column of silicic acid. Five percent ethyl ether was employed in the top reservoir with a lipid charge of 305.1 mg.

Chromatography of Membrane Lipids

As has been stated, a thorough understanding of the lipid content of the milk fat-globule membrane has eluded the investigation of many researchers. Fortunately, in this era of vast research facilities, the author had the benefit of chromatographic techniques. Thus, the separation of membrane lipids was accomplished employing silicic acid chromatography.

Table 3 and Figure 17 show data representing the elution characteristics of the various classes of lipids constituting the milk fat-globule membrane. Squalene, alpha-tocopherol, and cholesterol were not recovered quantitatively from the silicic acid column, an observation which reflects the fact that chemically pure (C. P.) grade compounds are not necessarily chromatographically pure since they were not quantitatively recovered from the column. The remaining lipids of the standard mixture were recovered quantitatively, and in some cases greater than 100% recovery was noted. The overall elution pattern differed from that reported by Hirsch and Ahrens (1958). This deviation might be attributed to the absence of nitrogen pressure on the column along with mechanical manipulations which contribute to elution changes. Nevertheless, this feature (i. e. , difference in area of elution) is inconsequential in view of the reproducibility and resolution characteristics of the elution patterns.

Figures 18, 19, and 20 show infrared spectra of the compounds

used as standards. These data were collected using carbon tetrachloride as the lipid solvent. Recognition of the fact that carbon disulfide is an excellent infrared solvent has not been avoided, and in many cases was employed for comparative purposes. The polystyrene spectrum in Figure 18 (D) assisted in locating and assigning wavelength absorption maxima of functional groups. The Beckman IR-5 was capable of sufficient resolution to adequately identify the lipid fractions.

Figure 21 represents a typical elution pattern for butteroil. Quantitatively, 100% of the lipid charge was recovered from the column. In two trials on the elution of butteroil, prepared by a modified Mojonnier extraction method, values of 3.44% and 3.25% cholesterol were obtained. These values are much higher than the ordinary range of 0.30 - 0.40%. No satisfactory reason can be given to explain this discrepancy although the values are evidently far too high. The carotenoids (peak 1) and cholesterol (peak 4) are indigenous to butteroil. Although small amounts of phospholipids appeared in the elution diagram, they are not reported here. The gross heterogeneity of the butteroil triglyceride, as evidenced by the multiple peaked triglyceride elution area (peak 3), is not surprising in view of the numerous combinations of mixed triglycerides which exist in butteroil. Peak 2 remains unassigned, but could be squalene. In contrast to the reported data of Jensen and Morgan (1959), it was impossible to detect mono and/or diglycerides in butteroil.

In contrast with the data obtained from butteroil are data obtained by silicic acid chromatography of the entire membrane lipid as outlined in Figure 3, scheme I. Figures 22 and 23 represent chromatograms of membrane lipids from two different membrane preparations varying only in the quantity of lipid charged on the column. This variation served to evaluate the resolving characteristics of the silicic acid column. The elution curves suggest the following interpretations. (a) The homogeneity of elution of the triglyceride of the membrane lipid, as opposed to butteroil, is striking. Such elution characteristics are suggestive of a relatively even distribution of fatty acids within the triglycerides. This conjecture could be examined by directed inter-esterification of the triglycerides, and by observing the variation in the melting range. Similar elution data on the HMGFractions (Figures 15 and 16) showed essentially identical characteristics. (b) The similarity in the elution characteristics of the HMGF and of the whole-membrane lipid triglycerides, along with the observation made by Thompson et al. (1959a) that the ethanol supernatants from HMGF crystallizations contained only traces of fatty acids, present sufficient evidence to warrant confirmation of the conjecture that the entire membrane tri-glyceride is composed of HMGF.

(c) The presence of mono-diglycerides (peaks 6 and 7) appearing in both chromatograms possibly could be accounted for on the basis of their orientation on the fat membrane interface as a result of their

surface activity. Likewise, lipolysis occurring during preparation of the lipid fraction and/or spontaneous lipolysis occurring on the silicic acid column was possible. These lipid fractions are present in whole milk to a small extent (Jensen and Morgan, 1959), but their presence in high concentration in the surface layers (Tables 4 and 5) is at least suggestive of lipolysis. The presence of unesterified fatty acids lends support to the above contention.

The presence of carotenoids, cholesterol and its esters has been reported previously, as has the presence of phospholipids. Table 7 serves to compare literature values with data obtained in this study on the concentration of the above lipids.

There has been some question in academic circles as to the efficiency with which silicic acid, as described by Hirsch and Ahrens (1958), separates the three classes of phospholipids. The standard elution curve, and the elution curves for unknown membrane lipid fractions show this separation to be compatable with the data of Hirsch and Ahrens (1958). However, resolution of the phospholipid components was not as effective in this study. The yields of phospholipid fractions from this study were in accord with the data of Rhodes and Lea (1958) and Smith and Jack (1959).

(d) Although the column charge varied from 278 mg. to 471 mg. there seemed to be little difference in the resolving characteristics of the column, although yield variations occurred.

In accord with the hypothesis that a minimum of experimental manipulation and temperature fluctuation would retard lipolysis in the membrane lipids, a third chromatogram, Figure 24, was run with lipids obtained from uncooled, fresh milk (Figure 3, scheme II). The elution curve for the triglyceride fraction (peak 2) was extremely sharp. Only small amounts of unesterified fatty acids appear in the chromatogram. Mono and diglycerides still exist as lipid components in a concentration of 7.45%, representing a substantial reduction when compared with the data from scheme I (Figures 22 and 23, Tables 4 and 5). Similarly, a 3.43% reduction in the triglyceride content appeared in the data from scheme II as opposed to those from scheme I. Figure 24 shows that ethyl ether was effective in removing the carotenoids and squalene-like compound (tentatively shown to be squalene by infrared analysis), confirming the observation by White et al. (1954) that the carotenoids (and vitamin A) were loosely bound at the membrane interface. A similar reduction occurred in the content of cholesterol in scheme II whereas the cholesterol ester content remained unchanged. An 8% increase in phospholipid content occurred in scheme II, reflecting the effect of temperature manipulation on the de-sorption of phospholipid from the membrane. Likewise, the percentage of lipid in the whole membrane was 43.76%, whereas in scheme I the value was 67.51%. The percentage difference is manifested in the protein content of the membrane, scheme II retaining considerably more protein material during the isolation process.

Table 3

Yields of lipid classes eluted by silicic acid chromatography (standard curve)

Compound	Sources	Charge (mg.)	Recovery (mg.)	Percentage recovered
Squalene	Nutritional Biochemicals	24.3	21.5	88.5
α-tocopherol (D-L)	Nutritional Biochemicals	31.4	24.5	78.0
Cholesterol	Nutritional Biochemicals	18.0	13.8	76.7
Cholesteryl palmitate	Delta Chemical Works	19.0	20.1	105.8
Stearic acid	Nutritional Biochemicals	18.3	19.1*	104.4
Monoglyceride	Eastman Distillation Products	21.9	22.7	103.7
Tripalmitin	Nutritional Biochemicals	18.6	20.2	108.6
Lecithin	Nutritional Biochemicals	21.5	21.8	101.4
Cephalin	Nutritional Biochemicals	19.0	21.8	114.7
Sphingomyelin	Nutritional Biochemicals	22.3	22.0	98.6
	Totals	214.3	207.5	96.8

Table 4

Composition of the lipid fraction of the milk fat globule membrane (scheme I), Figure 22

Lipid[a]	Peak number	Lipid eluted from silicic acid[b] (mg.)	Bound membrane lipid (%)
Carotenoids	1	3.0	1.11
Squalene		0	0
Cholesteryl esters	2	2.1	0.77
Triglycerides	3	132.3	48.93
Free fatty acids plus other glycerides	4	41.0	15.16
Cholesterol	5	7.2	2.66
Diglycerides	6	15.6	5.77
Monoglycerides	7	13.3	4.92
Phospholipids	8, 9, 10	55.9	20.67
Totals		270.4	99.99
Percentage recovery		97.83	

[a] Listed in order of elution from silicic acid column

[b] Silicic acid column as described by Hirsch and Ahrens (1958)

Table 5

Composition of the lipid fraction of the milk fat-globule membrane (scheme I), Figure 23

Lipid[a]	Peak number	Lipid eluted from silicic acid (mg.)[b]	Bound membrane lipid (%)	Whole membrane (%)	In milk fat (mg./100 g.)
Carotenoids	1	2.2	0.45	0.30	2.6
Squalene[c]	2	3.0	0.61	0.40	3.5
Cholesterol esters	3	3.9	0.79	0.54	4.6
Triglycerides	4	263.0	53.41	36.12	317.5
Free fatty acids plus other glycerides	5	31.0	6.30	4.26	36.2
Cholesterol	6	25.4	5.17	3.50	29.7
Diglycerides	7	40.0	8.14	5.49	46.7
Monoglycerides	8	22.9	4.66	3.14	26.7
Phospholipids	9, 10, 11	100.0	20.35	13.76	126.9
Totals		491.4	99.88	67.51	584.4
Percentage recovery		104.3			

[a] Listed in order of elution from silicic acid column

[b] Silicic acid column as described by Hirsch and Ahrens (1958)

[c] Precise identification still pending

Table 6

Composition of the lipid fraction of the milk fat-globule membrane (scheme II), Figure 24

Lipid[a]	Peak number	Lipid eluted from silicic acid (mg.)[b]	Bound membrane lipid (%)	Whole membrane (%)	In milk fat (mg./100 g.)
Squalene		0	0	0	0
Carotenoids		0	0	0	0
Cholesterol esters	1	2.9	0.63	0.27	4.4
Triglycerides	2	230.9	49.98	21.88	352.3
Cholesterol	3	16.8	3.64	1.59	25.6
Diglycerides	4	48.9	10.58	4.63	74.6
Monoglycerides	5	29.8	6.45	2.82	45.5
Phospholipids	6, 7, 8	182.7	28.72	12.57	202.5
Totals		462.0	100.00	43.76	704.9
Percent recovery		104.6			

[a]Listed in order of elution from silicic acid column

[b]Silicic acid column as described by Hirsch and Ahrens (1958)

Table 7

Percentage lipid composition of the milk fat globule membrane as compared to literature values

Lipid	Mg. / 100 g. fat		Literature values[a]
	Scheme I	Scheme II	
Carotenoids	2.6	0	0.0645% of membrane (White et al., 1954)
Vitamin A	---[b]	0	0.0483% of membrane (White et al., 1954)
Squalene	3.5	0	---
Cholesterol esters	4.6	4.4	15% of total cholesterol (Mulder and Zuidhof, 1958b)
Triglycerides	317.5	352.3	40.4 - 50.7%[d] of membrane (Rimpila and Palmer, 1935)
Unesterified fatty acids	36.2	----[c]	---
Cholesterol	29.7	25.6	36 mg. ± 16 mg. / 100 g. fat globule (Mulder and Zuidhof, 1958b)
Diglycerides	46.7	74.6	---
Monoglycerides	26.7	45.5	---
Phospholipids	126.9	202.5	0.17 - 0.29 g. / 100 g. fat (Rimpila and Palmer, 1935) 0.27 - 0.28 g. / 100 g. fat (Heinemann, 1939) 0.18 - 0.43 g. / 100 g. fat (Jenness and Palmer, 1945b)

[a] Does not imply that preparation procedures were identical

[b] Vitamin A and carotenoids would elute in the same area on silicic acid

[c] Unesterified fatty acids were difficult to accurately measure

[d] Listed as HMGF

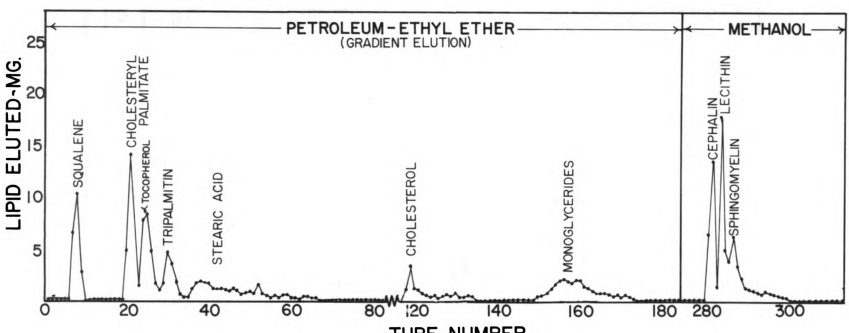


Figure 17. Elution of representative substances of eight major classes of lipids from an 18 g. column of silicic acid. Total lipid charge was 214.3 mg.

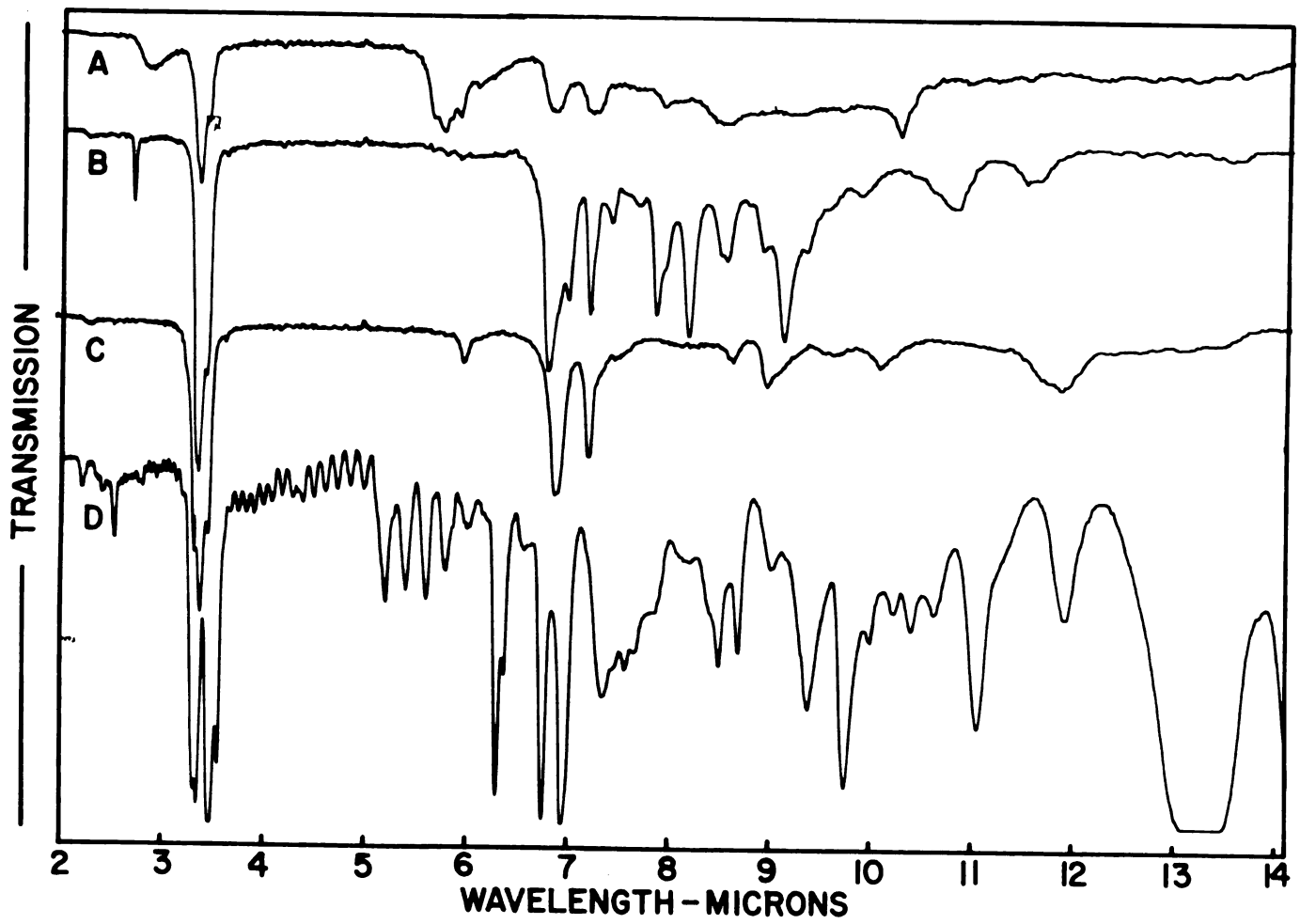


Figure 18. Infrared spectra of (A) beta carotene, (B) alpha tocopherol and (C) squalene carried in CCl_4 at 5% concentration. (D) represents polystyrene which was used as a standard for other compounds.

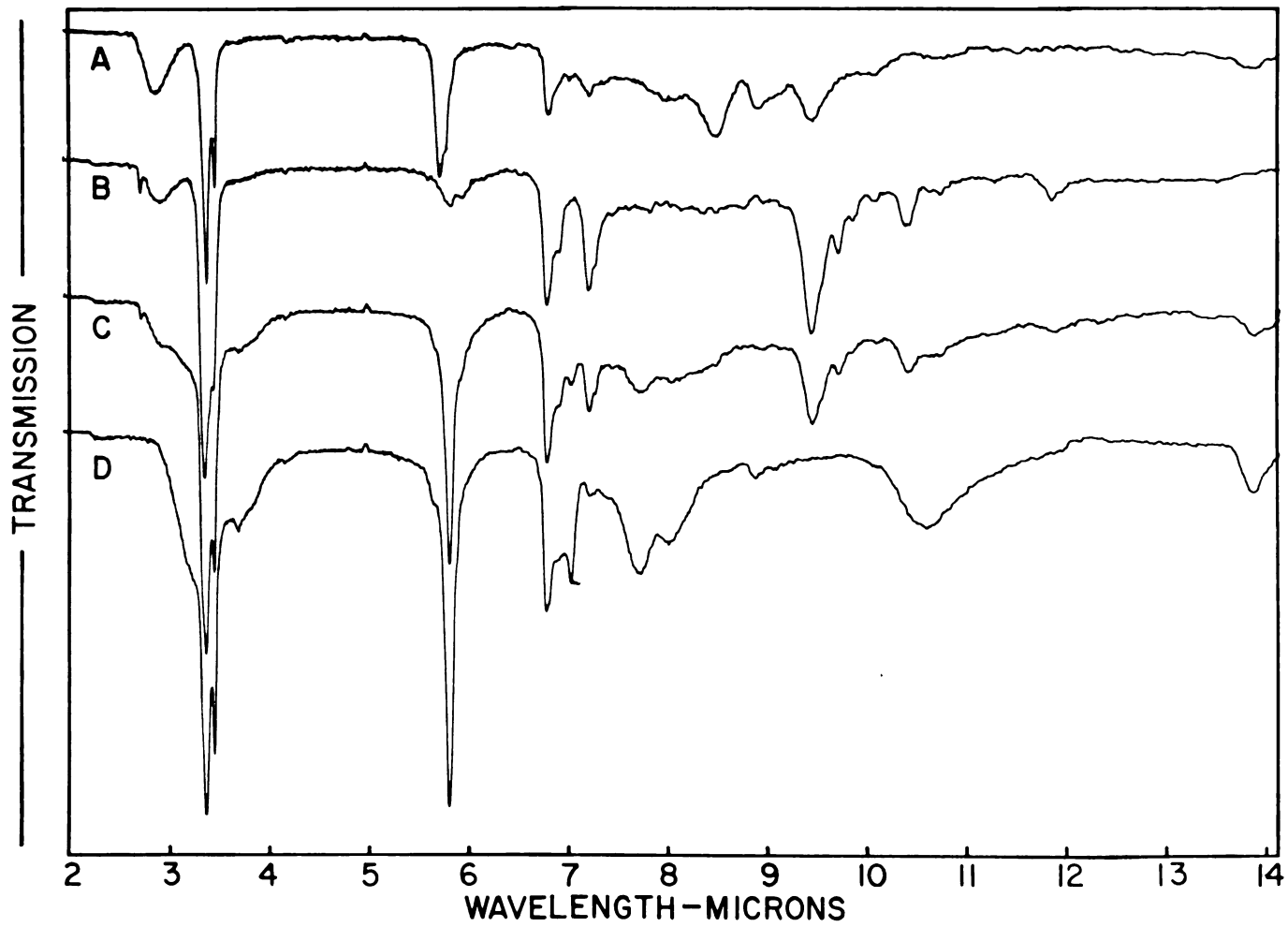


Figure 19. Infrared spectra of (A) monoglycerides, (B) cholesterol, (C) cholesteryl palmitate and (D) stearic acid carried in CCl_4 at 5% concentration.

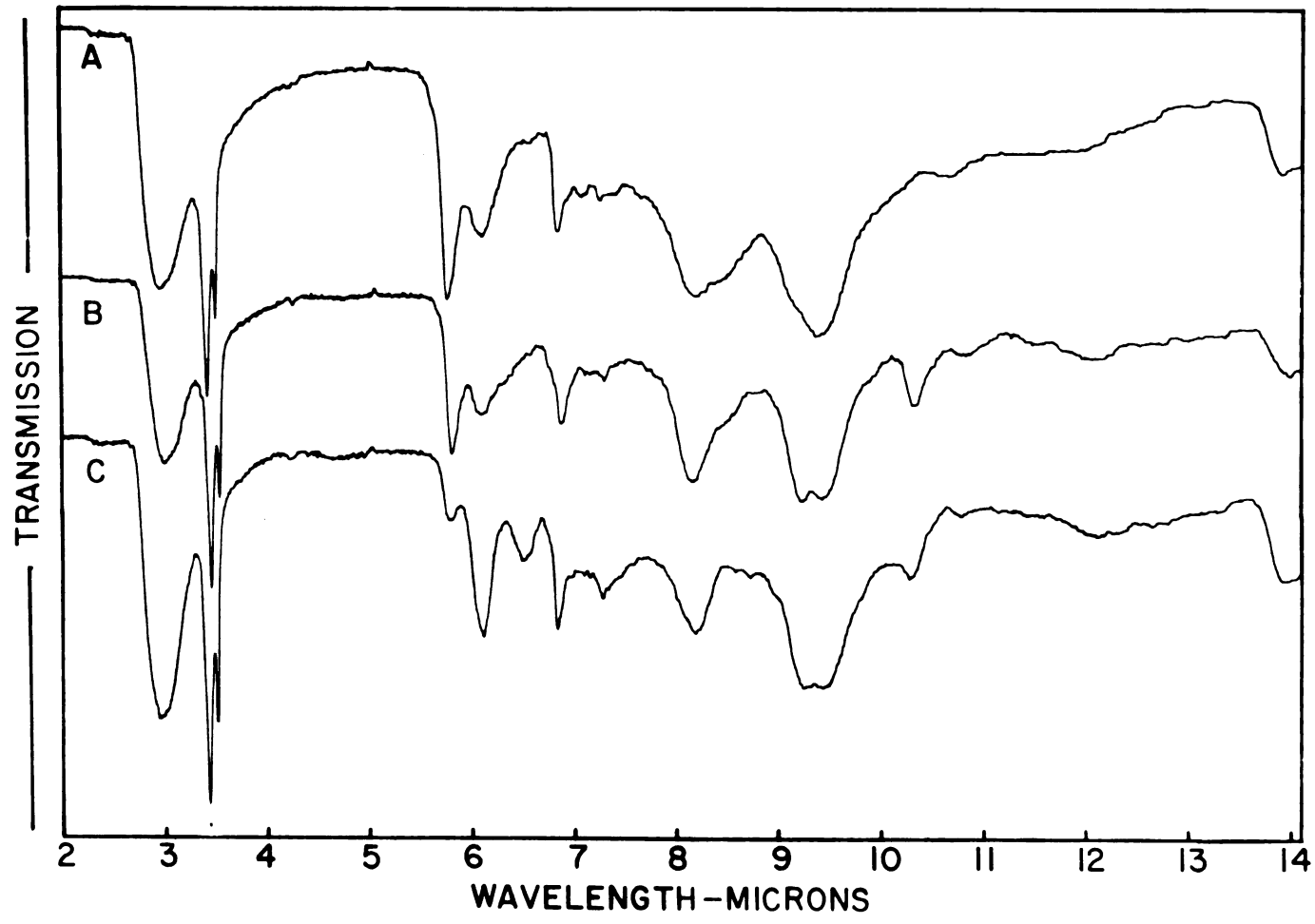


Figure 20. Infrared spectra of (A) cephalin, (B) lecithin and (C) sphingomyelin carried in CCl_4 at 5% concentration.

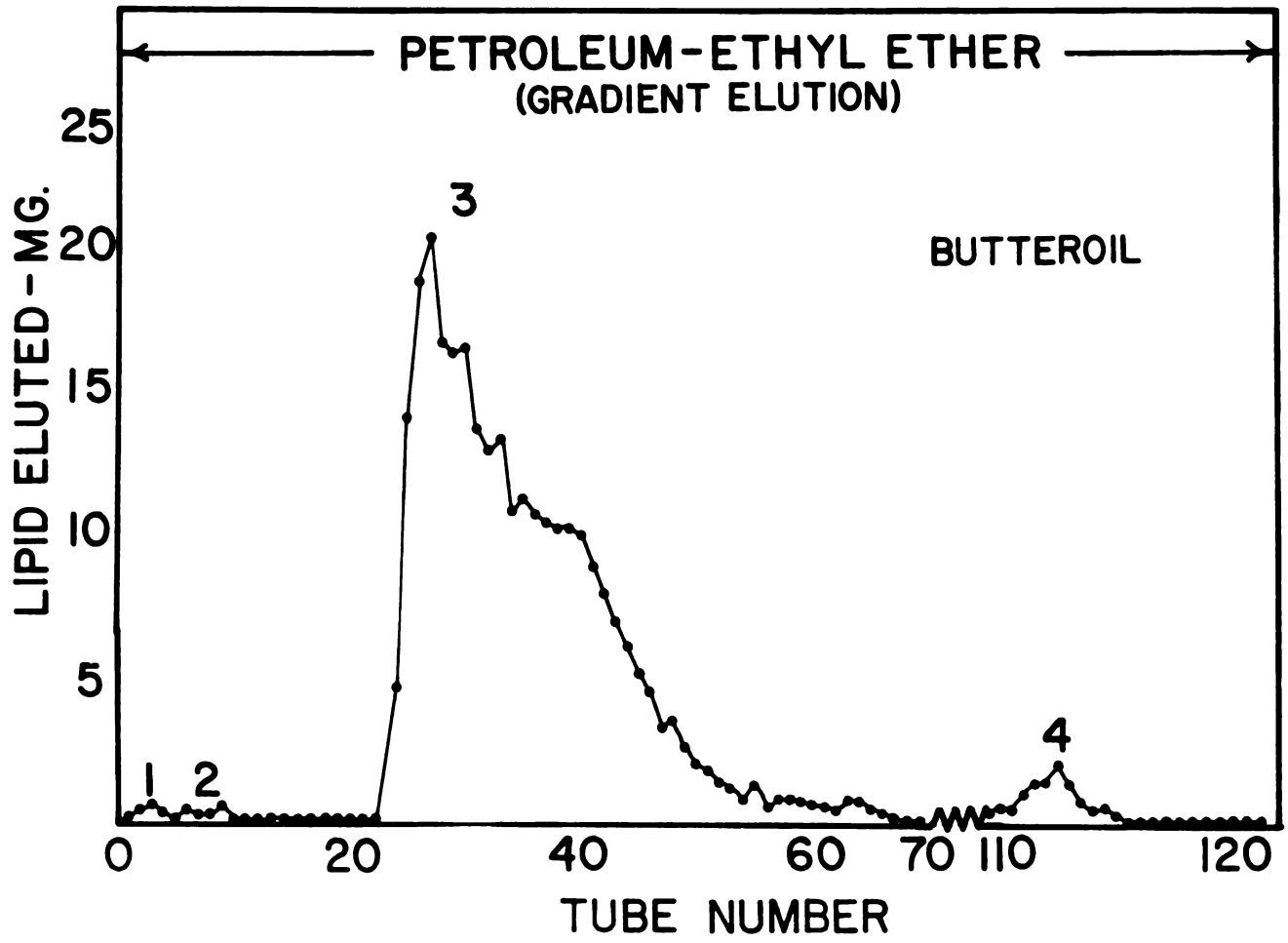


Figure 21. The separation of lipid components of butteroil by elution from an 18 g. column of silicic acid. Total lipid charge was 424.9 mg.

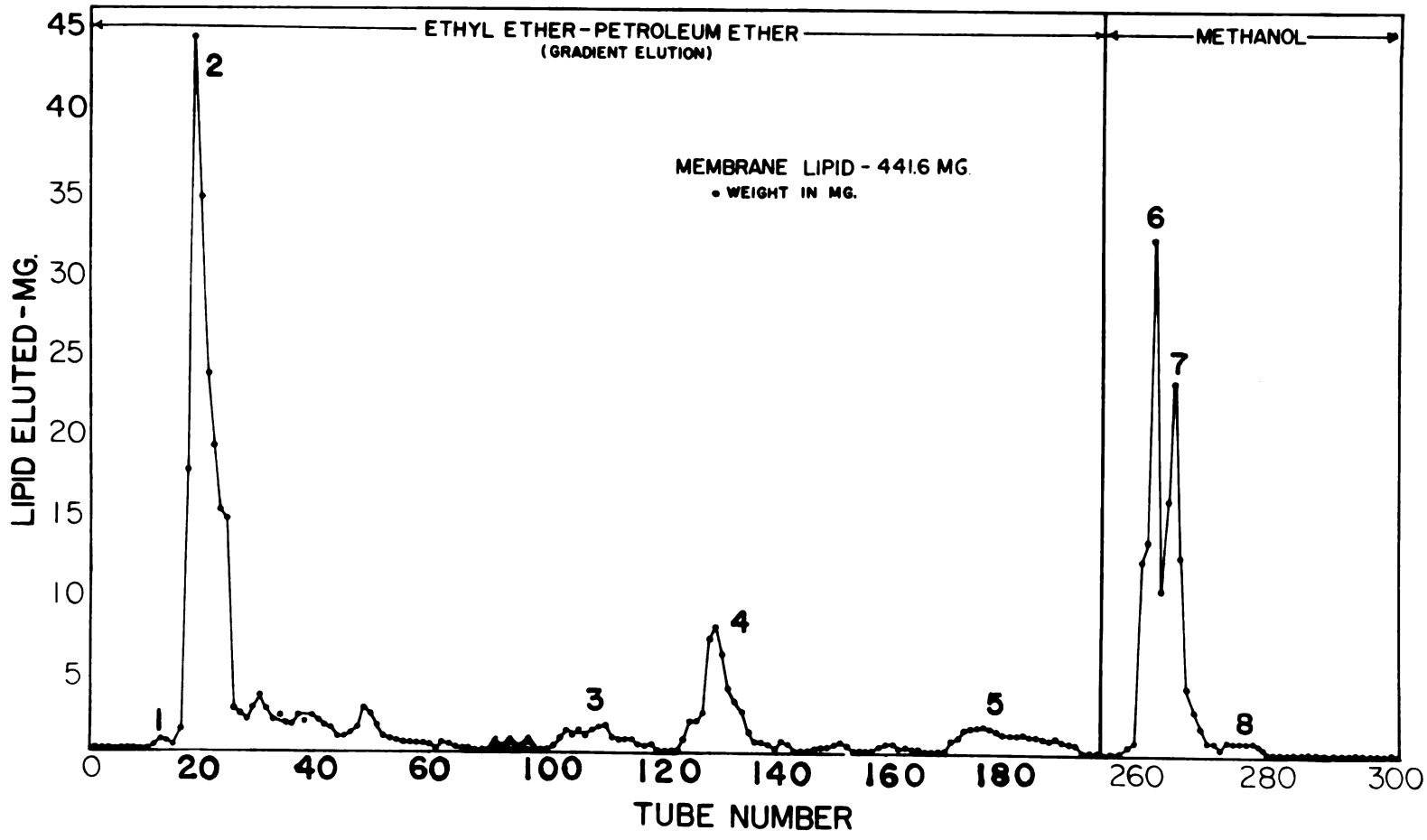


Figure 22. The separation of lipid components from the milk fat-globule membrane (scheme I) eluted from an 18 g.. column of silicic acid. Lipid charge was 280. 0 mg.

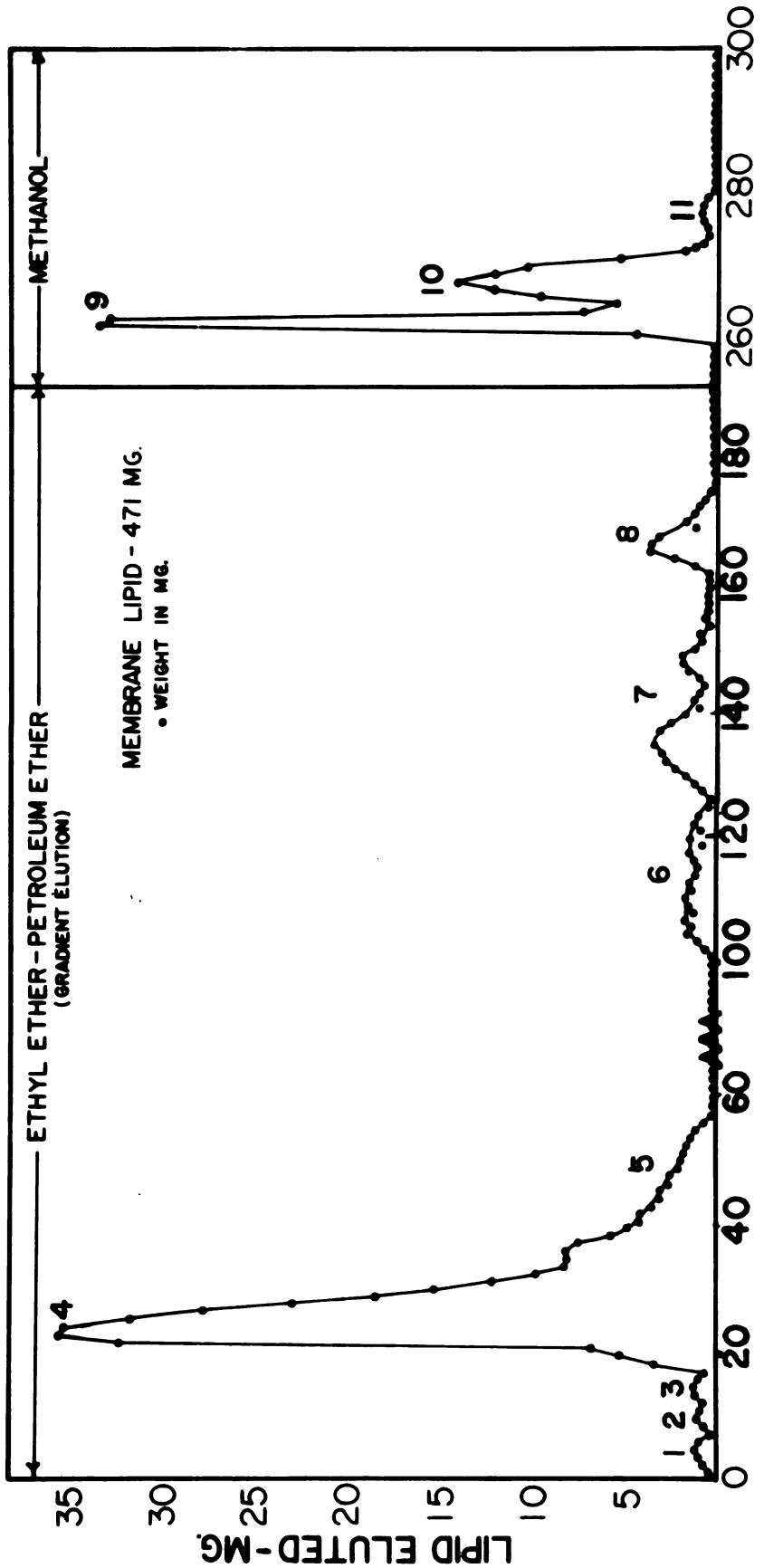


Figure 23. The separation of lipid components from the milk fat-globule membrane (scheme I) eluted from an 18 g. column of silicic acid. Lipid charge was 480 mg.

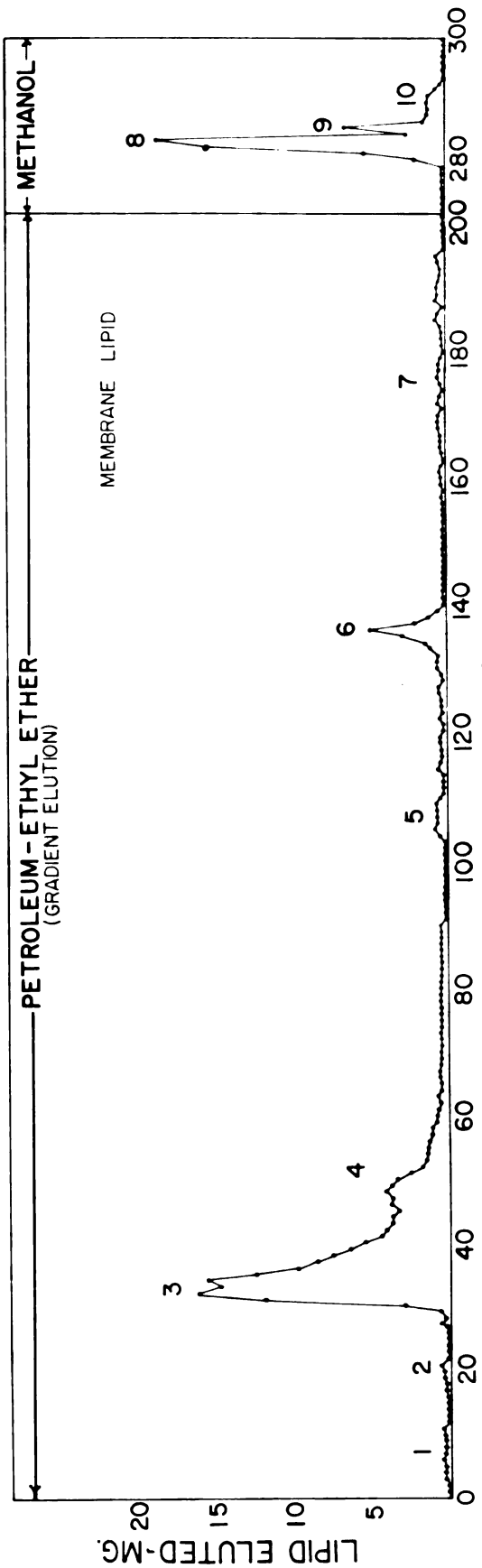


Figure 24. The separation of lipid components from the milk fat-globule membrane (scheme II) eluted from an 18 g. column of silicic acid. Lipid charge was 450 mg.

Soluble and Insoluble Proteins of the Fat Membrane

This portion of the study was directed to the characterization of the soluble and insoluble membrane-proteins as described by Herald (1956) and Herald and Brunner (1957). The success of any isolation procedure depends upon the reproducibility of the procedure. Thus, repeated isolations of these protein fractions were deemed necessary to determine yields of total membrane, membrane protein, and membrane lipids. Further, the physical and chemical nature of the soluble membrane-protein was compared with other minor proteins isolated from milk in an attempt to determine their similarities or differences.

A repetition of the experiments of Herald (1956) yielded total membrane material ranging from 0.94 g./100 g. fat to 1.61 g./100 g. fat depending upon whether cooled or fresh, warm milk was used for the isolations. When uncooled milk was analyzed, a total of 1.61 g./100 g. fat was obtained of which 0.70 g. was lipid, and 0.91 g. was protein. Cooled, pooled milk yielded 0.94, 1.28 and 1.40 g. of membrane/100 g. fat in three trials. The lipid contents of these three samples amounted to 0.55, 0.57 and 0.68 g./100 g. fat, respectively, whereas the protein concentrations were 0.39, 0.71 and 0.73 g./100 g. fat. Thus, it appears obvious that the total quantity of membrane lipid obtained per 100 g. fat was a relatively constant value whereas the protein content was the variable portion of the membrane substance.

The above yields correspond closely with those reported by Herald and Brunner (1957) who reported 1. 27 g. membrane/100 g. fat. Jenness and Palmer (1945b) report a range of 0. 71 to 1. 20 g. membrane/100 g. fat. Palmer and Weise (1933) had reported yields ranging from 0. 66 to 0. 89 g. membrane/100 g. fat. The yields reported here, then, are in good agreement with Palmer and associates, Herald and Brunner (1957) and Rosenberg (1957) who reported values ranging from 1. 14 to 1. 44 g. membrane/100 g. fat. The relative constancy of the literature values is a reflection of the method of preparation of the membrane--washing of cream.

In regard to the protein content of the membrane material, Herald and Brunner (1957) reported a value of 0. 51 g. protein/100 g. fat which amounted to 40% of the total membrane substance. Rosenberg (1957), following the membrane isolation procedure of Herald (1956), reported 0. 59 g. protein/100 g. fat. Earlier, Rimpila and Palmer (1935) reported values ranging from 0. 46 to 0. 71 g. protein/100 g. fat, and Jenness and Palmer (1945b) reported values of 0. 38 to 0. 86 g. /100 g. fat which is in excellent agreement with these observations. Mulder and Menger (1958a), employed gravity separation and washing as a means of procuring membrane material and reported 0. 1 to 3. 0 g. protein/100 g. fat which is an extreme range of protein yields as opposed to other reported data.

Thus, the protein yields contained herein correspond to the upper limits of the results obtained by the Palmer group. With one exception,

the protein obtained was greater than the lipid recovery, whereas Herald and Brunner (1957) observed the converse to be true. There is little question that the yield of membrane material depends upon (a) temperature history--cooling as opposed to non-cooling of milk, (b) agitation--method and number of washings, and (c) age of milk used for membrane preparations.

Sixty-one and six-tenths percent of the membrane-protein was determined to be soluble protein, whereas 38.4% was insoluble protein. Results from another isolation revealed values of 59.7% and 40.3% soluble and insoluble protein, respectively. Although Herald and Brunner (1957) separated soluble and insoluble membrane-proteins in 0.02 M sodium chloride, it should be emphasized at this time that the insoluble membrane-protein material could be centrifuged from the whole membrane "buttermilk" at 25,000 x G for one hour or less. This observation reflects the possibility that churning of cream frees the insoluble protein from the membrane complex.

Soluble and Minor Protein Fractions

Little data have been presented to substantiate a clear-cut classification of the soluble membrane-protein. Herald and Brunner (1957) stated that the fraction reacted positive to the Molisch test which is suggestive evidence for the presence of carbohydrates. Thompson and Brunner (1959c) showed that the soluble membrane-protein contained galactose, hexosamine, and neuraminic acid with a positive

reaction for fucose occurring. Nitschmann et al. (1957) earlier reported the presence of large amounts of carbohydrates in a scission product (obtained first by Alais, 1956) of the rennin reaction on whole casein. Brunner and Thompson (1959) characterized the glycomacropeptide from the reaction of rennin on casein.

Observations in this laboratory suggested that the soluble membrane-protein resembled the minor protein fractions isolated from skimmilk. It was noted that electrophoretic mobilities, non-coagulability of the proteins at high temperature, and glyco-protein nature of all the proteins were common properties of the fractions. Consequently, experimentation was initiated to compare the soluble membrane-protein with the minor protein fractions of Jenness (1959), Rowland (1938), Aschaffenburg (1946) and Weinstein et al. (1951a).

The composition of the minor protein fractions, Table 8, shows general similarities. The low nitrogen content and the presence of carbohydrates support their classification as glycoproteins. A previous report by Thompson and Brunner (1959c) showed that neuraminic acid was a constituent of these fractions. Each fraction contained relatively high concentrations of phosphorus and were correspondingly high in ash. The lipid, found in the proteose-peptone and soluble membrane-protein fractions represents residual lipid material not removed in the preparative procedure. The lipid is complexed in the fat-globule membrane, and as such is extremely difficult to remove in its entirety during the isolation of the soluble membrane-protein. An adjustment

of the compositional data, for this fraction, to correct for the 11.4% lipid would bring the nitrogen, phosphorus and hexose values more in line with those reported for the proteose-peptone fraction.

All of the protein fractions contained some 12% TCA-soluble protein, ranging from 4% for sigma-proteose to 41% for Weinstein's minor-protein fraction. Following treatment with crystalline rennin, the skimmilk fractions showed increases in the TCA-soluble protein. This was especially significant in the case of fractions receiving heat-treatment (95°C. for 30 minutes) at some stage in the isolation. The influence of heat-treatment on the TCA-solubility characteristics and on the capacity for rennin to act on the protein is demonstrated by the data for whey component 5 (Table 8), a fraction isolated by a procedure (Figure 8) not incorporating the heat treatment step normally employed for the other skimmilk-derived fractions. Approximately 6.6% of the original fraction was TCA-soluble. Following the addition of rennin to a water solution (pH 7.0) of the protein, 7.2% was TCA-soluble. This represents an increase of 9%. Interestingly, the heated solutions (95°C. for 30 minutes) contained 11.3% TCA-soluble proteins and, following the addition of rennin to the cooled solution, the TCA-soluble material increased to 14.8%, an increase of approximately 31%. This observation and the data of Jenness (1959), showing that heated whey component 5 would not depress loaf-volume when added to bread flour while the unheated fraction did so, suggests that the fraction or

a component thereof is heat-altered. Rennin did not act on the membrane fraction even when heated.

The high concentration of TCA-soluble protein found in the Weinstein fraction reflects the presence of scission products resulting from the action of rennet on casein (Alais, 1956), Nitschmann et al. (1957) and Brunner and Thompson (1959). These materials constitute approximately one-half of the Weinstein fraction and, if prepared from acid whey, the sigma-proteose fraction (Aschaffenburg, 1946).

Figures 25 and 26 represent free-boundary electrophoretic patterns of the minor-protein fractions in veronal, pH 8.6 and HCl-NaCl, pH 2.4 buffers, respectively. Electrophoretic mobilities and relative areas for the resolved peaks appearing in both the ascending and descending legs in these patterns are tabulated in Table 9. Although differences exist in the electrophoretic characteristics of these fractions, some similarities prevail which seem to be common to all the patterns. This is especially true for the characteristics of the major electrophoretic peak. Any further attempt to associate a specific peak appearing in another pattern would be hazardous in view of the electrophoretic complexity of the patterns. Consequently, the patterns are presented primarily for the purpose of demonstrating the overall electrophoretic differences and/or similarities of the various fractions in two buffers, differing widely in composition and pH. Nevertheless, the temptation to engage in some speculative interpretation of these data cannot be

denied. The proteose-peptone fraction, representing the total protein residue of heated skimmilk from which casein was removed by isoelectric precipitation, shows three peak areas. The sigma-proteose fraction, representing the heat stable protein, salted out of heated, acid whey with $(NH_4)_2SO_4$, shows only small concentrations of the leading peak as opposed to proteose-peptone. The relative absence of this fast moving component was also made for the Weinstein minor protein-fraction when run in veronal buffer. These observations show that a fast moving component, possibly of high phosphorus content with a mobility similar to whey component 8 was at least partially soluble in the 0.5 saturated $(NH_4)_2SO_4$ solution. This feature was demonstrated when proteose-peptone was salted out with 0.5 saturated $(NH_4)_2SO_4$. Although the leading component was not completely removed its concentration was markedly reduced. The closely associated pair of peaks in the Weinstein fraction (in veronal buffer) represent the major proteose-peptone component and the glyco-macropeptide (GMP), released by the action of rennin on casein (Nitschmann et al., 1957). The heterogeneity of this fraction was most obvious in acid buffer, reflecting the action of rennet on casein and possibly on the proteose-peptone itself.

The Jenness fraction shows at least four electrophoretically discernible components. Examination of a fraction obtained from Dr. Jenness showed 7 components in the ascending boundary. Obviously, then, whey component 5 is not the only protein present in this fraction.

The fraction prepared in this laboratory was contaminated by a small amount of beta-lactoglobulin, which appears in the sedimentation diagram in the position normally assigned for this protein. Interestingly, the eluate (pH 2.5) from the IRC - $50H^+$ column, employed in the removal of lactoperoxidase from this fraction, contained a floc which was not apparent at the same pH in the protein solution prior to its passage over the column. It is conceivable that the column-retained components serve to stabilize the entire system. It is interesting to note that the IRC - $50H^+$ retained protein material was rich in whey component 8 (unpublished data). Experiments were in progress at the writing of this thesis to characterize components 3, 5 and 8 of the whey serum pattern. Compositional and electrophoretic characteristics (in veronal buffer) of the soluble membrane-protein were approximately similar to those of the skimmilk fractions.

Figure 27 and Table 10 show sedimentation-velocity diagrams and coefficients (S20, uncorrected), respectively for the minor protein fractions. The sedimentation diagrams of the skimmilk derived proteins exhibited a common, major sedimenting boundary of about 0.8 Svedburgs and, in the heated fractions, a faster moving minor boundary of about 2.8 Svedburgs. The whey component 5 diagram exhibited a fast moving component of $S_{20} = 5.88$ which is close to that of beta-lactoglobulin at the same pH. The diagrams for the soluble membrane-protein reflect molecular size differences between this fraction and those previously

mentioned. This observation suggests that the minor whey proteins and the soluble membrane-protein are not identical at least by ultracentrifugal analysis.

The whey component 5 and soluble membrane-protein are isolated by methods not employing the use of high temperatures. For the purpose of making a more comparable evaluation of these fractions with those in which exposure to high temperatures constituted a step in the preparation, water solutions of these two fractions were heated to 95° C. for 30 minutes, cooled, filtered, dialyzed against buffer and reexamined electrophoretically and ultracentrifugally (Figures 28 and 29, Table 11). Heating the protein fractions resulted in a general decrease in the electrophoretic mobilities of the constituent components. Similarly the heated minor proteins possess lesser mobilities than unheated whey component 5 in the initial study. Specifically, the Jenness fraction showed a reduction in the area of peaks 1 and 4 while the membrane protein showed an increased resolution in veronal buffer and a corresponding decrease in acid buffer. The sedimentation boundary ascribed to beta-lactoglobulin was absent from the sedimentation diagram of the Jenness fraction (Figure 29). But the new boundary, similar to the fast-moving boundary observed in the heat-isolated fractions (S_{20} = 2.8) appeared. The sedimentation boundaries of the membrane fraction were more discernible and of somewhat lower values, indicating the dissociating influence of heat on the fractions.

Before concluding the subject of the minor proteins of milk, several observations should be made clear. First, the soluble membrane-protein is not in its normal state because it was obtained by ethanol-ether extraction methods from intact membranes. The protein probably serves in the capacity of acting as a polypeptide backbone for the membrane lipoprotein. Thus, comparing it with the true minor protein fraction of milk is hazardous. Secondly, the minor-protein fraction of Weinstein is not a normal milk constituent in its entirety; it was obtained from rennet treated skimmilk. The writer is firmly convinced that any property ascribed to this fraction in reference to normal whole milk is in error, hazardous, and a breach of honest research. The fraction unquestionably contains rennet scission products from whole casein.

Thirdly, heat preparation of the minor proteins is not to be recommended in preparation of the minor proteins for characterization studies. Any heat effect, although minor, is sufficient to alter the physical properties of the constituent proteins. Nor is the procedure of Jenness (1959) recommended for securing whey component 5 in a pure state. And, in fact,the salting-out method introduces protein fractions which are non-existent in the proteose-peptone and sigma-proteose fractions. To secure pure components 3, 5, and 8 such chromatographic separations as DEAE-cellulose or Sephadex should be applied. Nevertheless, for obtaining gross minor protein fractions from skimmilk this procedure is recommended because it imparts no heat effects to the protein group.

Fourthly, the fact that sigma-proteose contains considerably less phosphorous and less component 8 than Jenness' fraction or the proteose-peptone fraction indicates that component 8 is rich in phosphorous, and may well be a nucleoprotein. Phosphorous determinations on the $(NH_4)_2SO_4$ soluble material from the Weinstein fraction showed a value of 10%. However, if phosphopeptides were released as a result of the rennin reaction they would, in all likelihood, appear in the ammonium sulfate supernatant. A pure nucleic acid would contain as much as 11% phosphorous on a molar basis. The possibility that component 8 is a nucleoprotein is a question of basic interest and of major interest in respect to Jenness' studies as regard loaf-volume.

Fifthly, whey component 5 is an unusual protein in respect to its solubility at pH 4.6 while in dilute solution. However, at high concentration (2-5%) it easily precipitates at pH 4.6. The true isoelectric point of this fraction is still questionable, but it is certain that it lies below pH 5.2 and above pH 4.2. The fraction might be termed a "serum casein" on the basis of its high phosphorous content, and general isoelectric point. Its high phosphorous content (1.2 - 1.5%), low sedimentation coefficient of less than 1 S are characteristic properties of lambda casein. This laboratory considers the minor protein fractions of great interest and importance, and will continue research directed toward their characterization.

Table 8

Composition of the minor protein fractions of bovine milk

Fraction	Component					Percentage of fraction soluble in 12% TCA solution	
	Nitrogen	Phosphorus	Ash	Hexose	Fat	Original	Rennin treated
	(%)						
Proteose-peptone (heat stable whey protein)	13.60 13.70-13.90	1.10	4.90-5.37	2.90-4.30	1.30	19.35	32.25
S-proteose	13.72-13.96	0.88	3.22			3.98	4.97
Component "5"	12.60	1.50	9.82	0.92	0	6.67	7.26
Heated "5"	12.60	1.50	9.82	0.92	0	11.30	14.78
Minor protein fraction	10.25-10.40	0.63	5.35-5.50	3.46-6.94	0	41.24	46.67
Soluble membrane protein	10.35	0.68	6.60	2.69-4.04	11.40	6.03	6.03

Table 9

Electrophoretic mobility and relative percentage area of the migrating boundaries of the minor protein fractions as determined from the descending-channel electrophoretic mobility patterns shown in Figures 25 and 26

Electrophoretic mobility patterns	Buffer system ($\Gamma/2 = 0.1$)	Migrating boundary[a] 1		2		3		4	
		μ[b]	A%	μ	A%	μ	A%	μ	A%
Figure 25	Veronal, pH[c] 8.6								
Row A[d]		7.4	17.2	4.5	79.3	2.7	3.5		
		6.3	28.0	3.5	66.7	2.0	5.3		
Row B[e]		6.6	3.0	4.5	87.8	2.6	9.2		
		5.8	7.0	3.9	85.5	2.0	7.4		
Row C[f]		8.8	4.2	6.5	6.1	5.4	39.0	3.6	50.8
		7.2	9.8	5.4	15.9	4.4	52.5	2.8	21.8
Row D[g]		5.4	56.4	4.8	38.7	3.2	4.6		
		5.3	65.6	4.5	30.4	3.1	4.0		
Row E[h]		4.8	97.4	2.2	2.6				
		4.7	96.5	2.3	3.5				

[a] Designation of boundaries shown in Figures 1 and 2

[b] Mobility (μ) = cm.^{2}, sec.$^{-1}$, v.$^{-1}$, x 10^{-5}; migrating distances measured from initial boundary

[c] pH values measured at 22°C.

[d] Proteose-peptone (Rowland, 1938)

[e] Sigma proteose (Aschaffenburg, 1946)

[f] Whey component "5" (Jenness, 1959)

[g] Minor protein fraction (Weinstein, et al. (1951a)

[h] Soluble membrane-protein (Herald & Brunner, 1957)

Table 9 (Continued)

Electrophoretic mobility patterns	Buffer system ($\Gamma/2 = 0.1$)	Migrating boundary[a] 1		2		3		4	
		μ[b]	A%	μ	A%	μ	A%	μ	A%
Figure 26	HCl-NaCl, pH 2.4[c]								
Row A[d]		5.6	2.3	4.5	4.6	3.4	58.5	2.5	34.6
		5.6	5.4	---	---	2.3	70.0	1.5	26.6
Row B[e]		4.0	56.4	3.1	74.4				
		3.0	43.6	1.7	25.6				
Row C[f]		8.7	8.8	6.4	12.9	4.3	68.3	3.4	9.9
		8.4	6.2	5.6	12.8	3.4	65.0	2.5	16.0
Row D[g]		7.6	10.7	4.1	17.0	3.0	24.6	1.8	16.3
		6.4	15.2	3.4	16.6	2.5	18.0	1.4	22.9
Row E[h]		4.5	78.1	2.8	15.6	1.9	6.3		
		4.3	76.4	3.4	14.6	1.4	9.0		

[a] Designation of boundaries shown in Figures 1 and 2

[b] Mobility (μ) = $cm.^2$, $sec.^{-1}$, $v.^{-1}$, $\times 10^{-5}$; migrating distances measured from initial boundary

[c] pH values measured at 22°C.

[d] Proteose-peptone (Rowland, 1938)

[e] Sigma-proteose (Aschaffenburg, 1946)

[f] Whey component "5" (Jenness, 1959)

[g] Minor protein fraction (Weinstein et al. (1951a)

[h] Soluble membrane-protein (Herald & Brunner, 1957)

Table 10

Sedimentation coefficients $(S_{20})^a$ for the minor protein fractions of bovine milk measured from the sedimentation-velocity diagrams shown in Figure 27

Fraction	Sedimentation boundary	
		(S_{20})
Proteose-peptone		
Row A	2.64	0.77
Sigma-proteose		
Row B	2.86	0.83
Whey component "5"		
Row C	5.88	0.88
Minor protein fraction		
Row D	2.83	0.84
Soluble membrane-protein		
Row E	17.60	9.05

$^a S_{20} = S_{20} \times 10^{-13}$; run in veronal buffer at pH 8.6, $\Gamma/2 = 0.1$; 1.5% protein conc.; uncorrected for buffer vixcosity.

Table 11

Compilation of electrophoretic and sedimentation-velocity values for whey component "5" and soluble membrane-protein following heat treatment at 95° C. for 30 minutes as determined from the data shown in Figures 28 and 29

Electrophoretic and sedimentation patterns	Buffer ($\Gamma/2 = 0.1$)	Electrophoretic boundary 1		2		3		4		Sedimentation boundary 1	2
		μ	A%	μ	A%	μ	A%	μ	A%	S_{20}	
Figure 28	(Electrophoretic)										
Row A	Veronal, pH 8.6	8.4	9.6	6.2	8.4	4.9	60.0	3.1	21.9		
		6.9	12.3	4.8	14.4	3.7	55.1	2.2	18.2		
Row B	Veronal, pH 8.6	4.7	63.3	3.7	27.0	3.3	8.3	1.7	1.4		
		4.7	70.8	3.4	27.9	2.5	2.2	---	---		
Row C	HCl-NaCl, pH 2.4	4.4	61.9	3.5	38.1						
		3.6	80.0	2.5	20.0						
Row D	HCl-NaCl, pH 2.4	3.3	100.0	---	----						
		3.3	59.1	2.3	40.9						
Figure 29	(Sedimentation)										
Row A	Veronal, pH 8.6									2.3	0.8
Row B	Veronal, pH 8.6									16.9	6.6

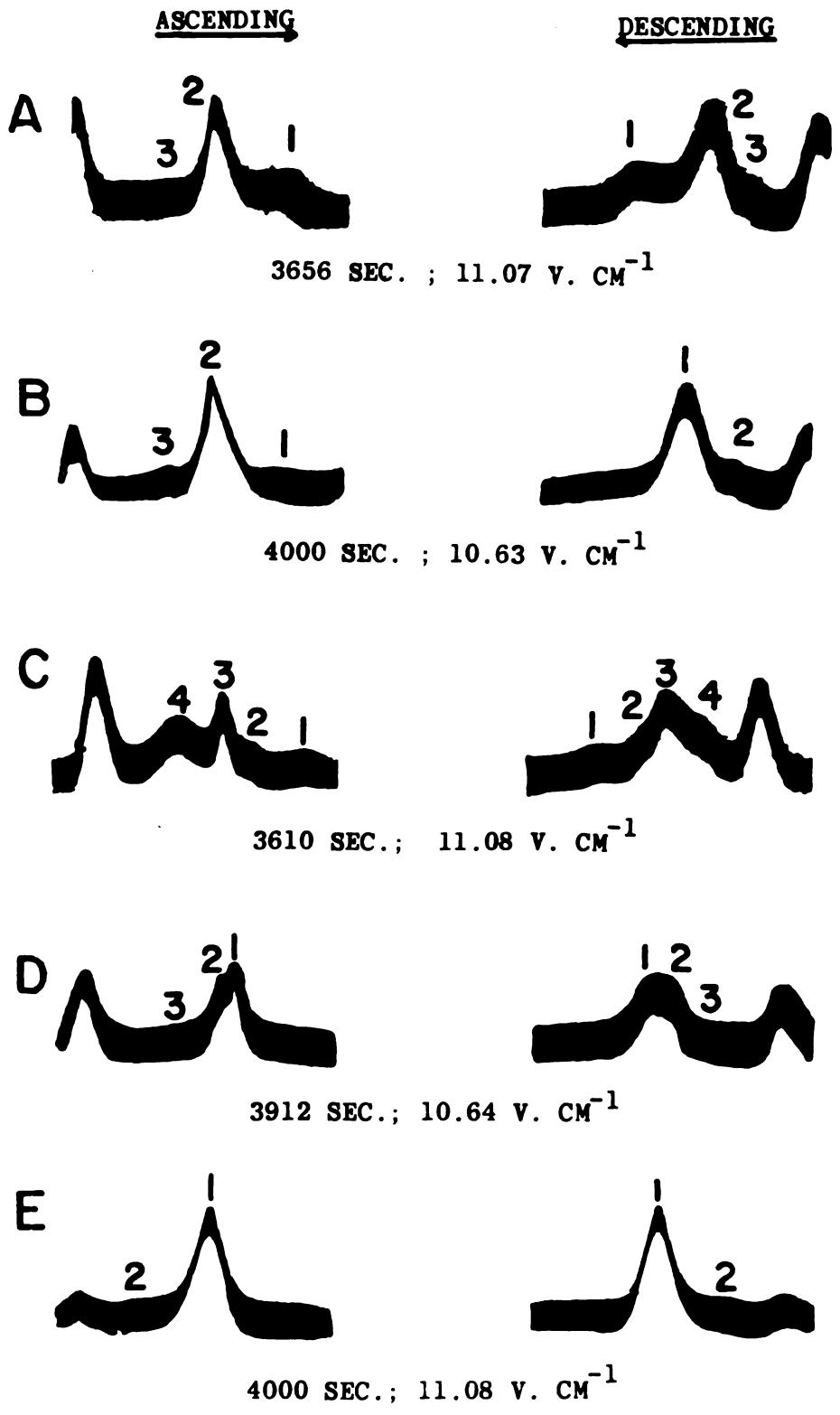


Figure 25. Electrophoretic patterns of (A) proteose-peptone, (B) sigma-proteose, (C) whey component 5, (D) minor-protein fraction and (E) soluble membrane protein. The fractions were run in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$ at 1.5% concentration.

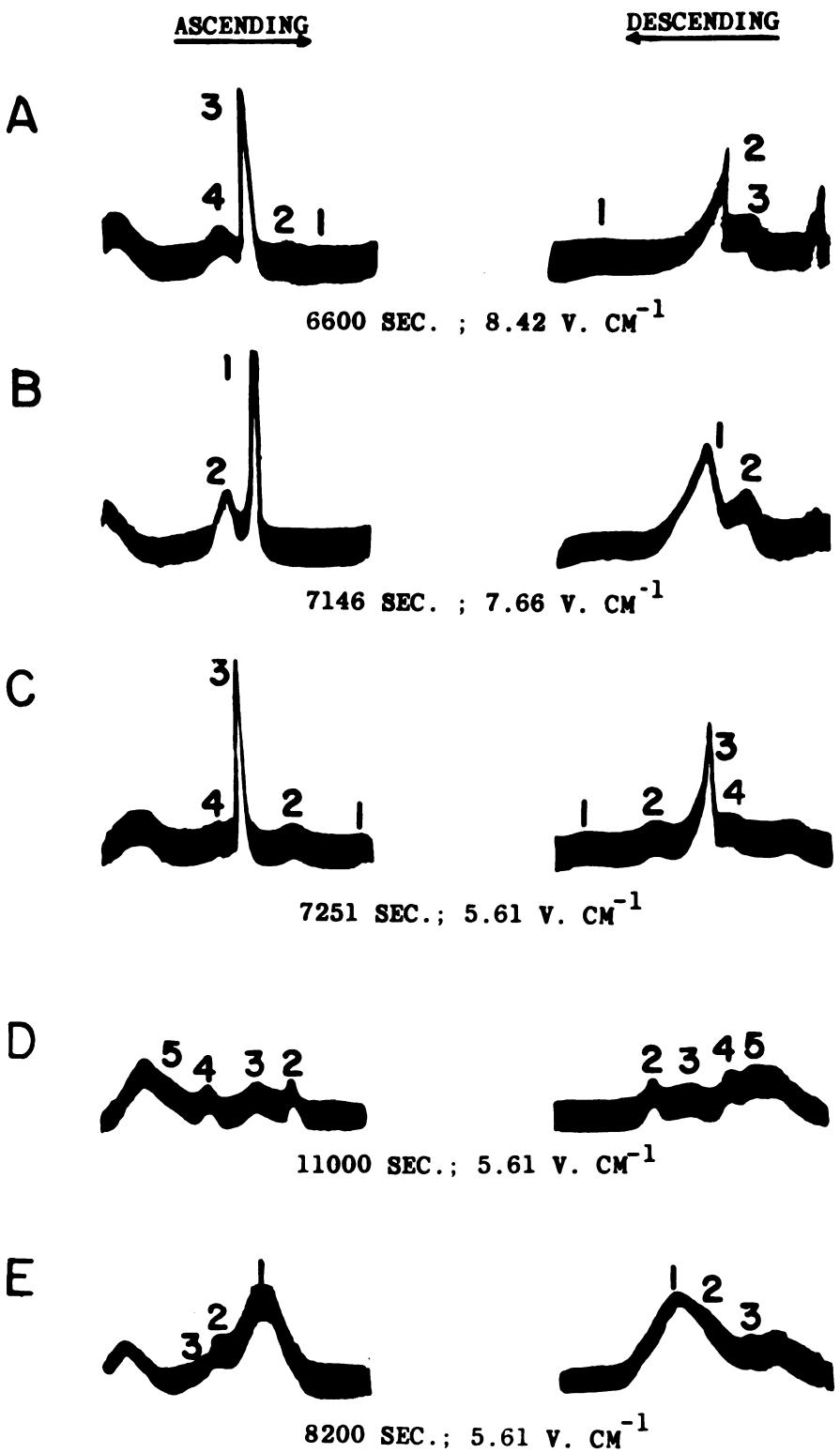


Figure 26. Electrophoretic patterns of (A) proteose-peptone, (B) sigma-proteose, (C) whey component 5, (D) minor-protein fraction and (E) soluble membrane protein. The fractions were run in HCl-NaCl buffer, pH 2.4, $\Gamma/2 = 0.1$ at 1.5% concentration.

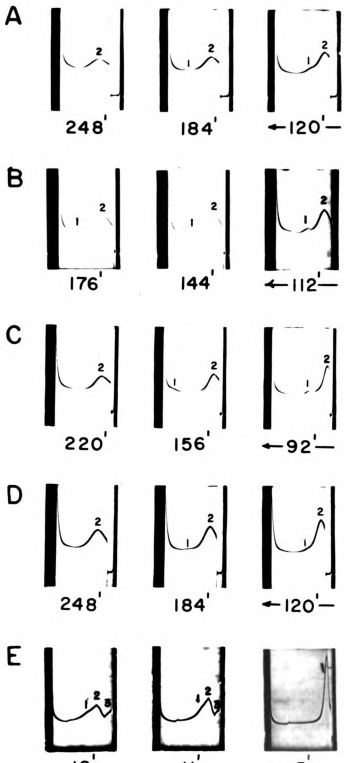


Figure 27. Sedimentation diagrams in veronal buffer, pH 8.6, $\Gamma/2$ = 0.1, 1.5% concentration of (A) proteose-peptone, (B) sigma-proteose, (C) whey component 5, (D) minor-protein fraction and (E) soluble membrane protein. All phase plate angles were at 60°. Rotor speeds for A-D were 59,780 r.p.m. and E. was 52,640 r.p.m.

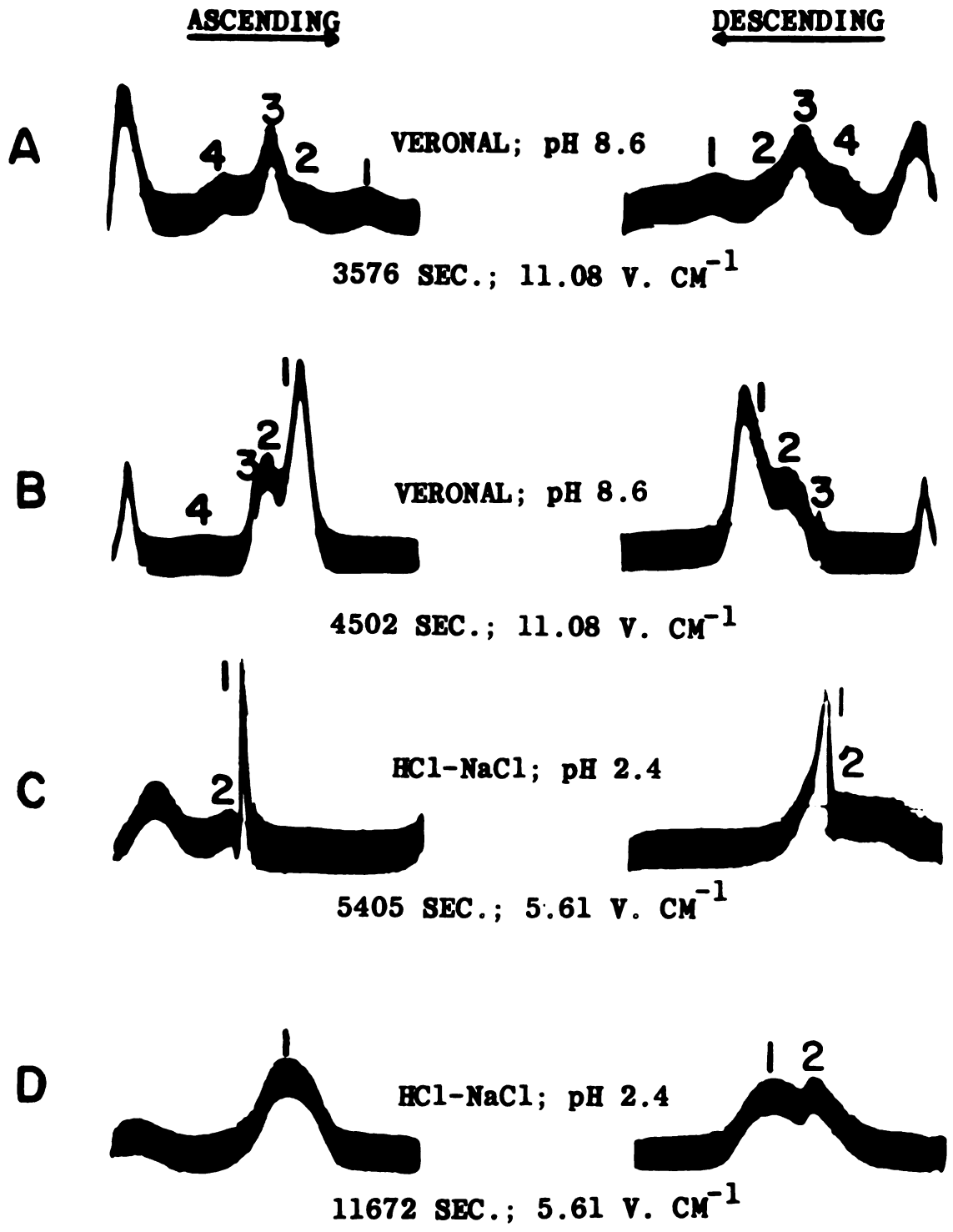


Figure 28. Electrophoretic patterns of (A) heated component 5, (B) heated soluble membrane protein, (C) heated component 5, and (D) heated soluble membrane protein in buffers as indicated at 1.5% concentration.

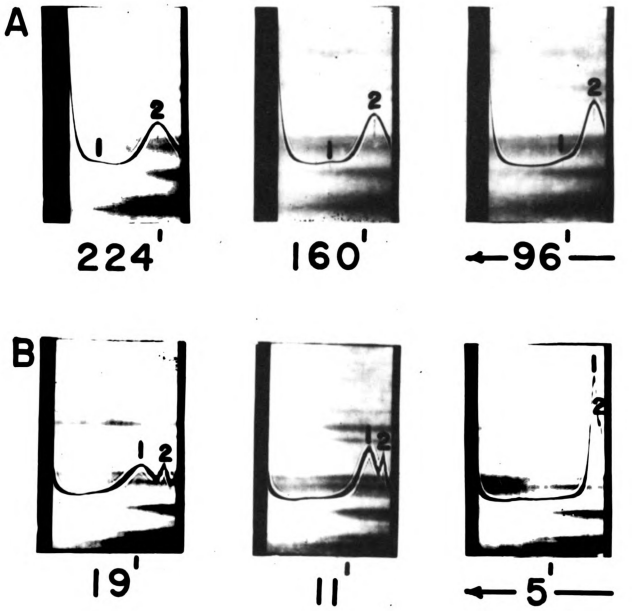


Figure 29. Ultracentrifugal diagrams in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$, 1.5% concentration of (A) heated whey component 5, and (B) heated soluble membrane protein. (A) was run at 59,780 r.p.m. with a phase plate angle of 60°, and (B) was run at 52,640 r.p.m. with a phase plate angle of 50°.

Lipoproteins

Since the ultimate in any protein research problem is to study the protein in as near the "native" state as possible this study was undertaken. Since considerable confusion exists as to the nature of the soluble membrane-protein, a considerable amount of emphasis is placed on comparing the soluble membrane-protein of Brunner and Herald (1957) with that of Ramachandran and Whitney (1960) as well as with the soluble fractions obtained from this study. It is the intent of this section to clarify the existing confusion on the nature of the membrane proteins, and to discuss the true lipoprotein of the fat membrane.

Figure 30 demonstrates the electrophoretic characteristics of the soluble membrane-protein of Brunner and Herald (1958) obtained by solvent extraction of the lipid. In veronal buffer, pH 8.6, this fraction possessed an electrophoretic mobility for peaks 1 and 2, of -4.33 and -1.95 respectively. For a similar fraction in the same buffer, Ramachandran and Whitney (1960) reported electrophoretic mobilities of -3.03, -4.23 and -5.41 for three peaks, the middle peak apparently comprising the major component. Correspondingly lower mobilities were observed for the migrating peaks at pH 7.2 in phosphated buffer, which were -4.12 and -1.73 from this study, whereas Ramachandran and Whitney (1960) report -2.37 and -1.65 at pH 6.9 in malonate buffer. A three component system was observed in glycine-HCl buffer, but evidence has been produced (proteins section) which suggests that

glycine complexes with protein fractions thus causing artifacts in the protein system. Electrophoretic characteristics of the soluble membrane-protein in HCl-NaCl buffer (pH 2.4) and glycine-HCl (pH 2.1) were grossly different. A comparison of the electrophoretic properties of the soluble membrane-protein obtained by three different researchers (Table 12) suggest that the soluble membrane-protein isolated for this study was similar, if not identical, to the fraction prepared earlier by Brunner and Herald (1958) and Ramachandran and Whitney (1960).

In the ultracentrifuge, the soluble membrane-protein showed sedimentation characteristics which appear to reflect the concentration of the lipid present in the fraction. As the lipid content was reduced, more homogeneity and symmetry was observed in the sedimenting boundaries. One preparation of the soluble membrane-protein, from this laboratory, containing 11.4% lipid showed two sedimenting boundaries (S_{20}, uncorrected of 17.60 and 9.05). Brunner and Herald (1956) reported values of 9.8 and 1.7 for sedimentation coefficients, whereas Jackson and Brunner (1960) reported $S_{20}^{c=o}$ of 6.0 and 2.7 for a soluble, heated membrane protein fraction. Numerous preparations of the soluble membrane-protein isolated in this laboratory showed identical electrophoretic properties, but dissimilar ultracentrifugal properties depending upon the lipid content of the fraction.

Ramachandran and Whitney (1960) reported one boundary with a sedimentation coefficient of 8.84 + 0.73. Possibly the variations observed in the sedimentation characteristics of these fractions can be ascribed to

variations in their lipid content which, conceivably, contribute to various degrees of molecular dispersion. The fact (Table 12) that the soluble membrane-proteins obtained by three researchers are practically identical in nitrogen and phosphorous contents strongly suggests that the fractions are identical. The fractions of Herald and Brunner (1957) and Thompson and Brunner (1959c) contained significant amounts of carbohydrate.

The insoluble membrane-protein is virtually insoluble in aqueous solvents (Brunner and Herald, 1958). However, solubilization of the fraction can be attained by any of the following methods plus those methods described by Brunner and Herald (1958): adjustment of the pH to 11.8 in water suspension, treatment with peracetic acid, and the use of 2' thiodiethanol. The ensuing solubilized protein, although atypical of its native state, was electrophoretically homogeneous (Figure 31). The proteins possessed electrophoretic mobilities of -3.95, -4.72 and -4.04, respectively, when solubilized at pH 11.8, with 3% peracetic acid and 2' thiodiethanol.

Low angle x-ray diffraction studies on unsolubilized insoluble membrane-protein suggests that the protein has a repeating unit and is definitely fibrous in nature. This feature will be considered further in this section.

Differential sedimentation of the membrane sera in 10% sucrose solution served as an excellent technique for fractionating these materials into three distinctly characteristic portions: a floating plug, a sedimenting

pellet, and an optically opaque center-cut. The sucrose was especially useful since it was a non-electrolyte. The pellet contained the insoluble membrane-protein fraction previously mentioned. An analysis of the extracted pellet-lipids (Figure 32 and Table 13) showed the presence of cholesterol, unesterified fatty acids, triglycerides and mono-diglycerides. In comparing the lipid fraction with the whole membrane lipid (Figures 22, 23) several features are evident: (1) the fraction is virtually free of cholesterol esters and the squalene-like compound. (2) The ratio of phospholipids to triglycerides has shifted from a 1:2 to a 1:1 ratio. (3) The ratio of lecithin to cephalin is nearly 1:1 and the fraction is devoid of sphingomyelins. The absence of carotenoids and the squalene-like compound suggest that the insoluble membrane-protein is at no time in direct contact with these lipids. Additionally, the unusual ratio of cephalin to lecithin and the absence of sphingomyelin indicates that the first two phospholipids are important in the complex of the insoluble membrane-protein with the remainder of the lipid-protein complex. The total lipid content of the insoluble membrane-protein was 8%.

In addition, when the insoluble membrane-protein was prepared by sucrose fractionation (with no solvent extraction) it could be dispersed and observed electrophoretically at pH 8.6 (Figure 33, C). An electrophoretic mobility of -4.15 for this fraction was practically identical to the solubilized fractions. In the ultracentrifuge, the protein completely sedimented out at 10,000 r.p.m.

The center-cut, which was opaque, possessed a lipid content (45.64%) similar to the entire lipid fraction (Figure 34, Table 13), but was richer in the squalene-like compound, cholesteryl esters, cholesterol and carotenoids, and substantially richer in mono-diglycerides. Probably then, this fraction constitutes the true outer layer of the membrane since surface active materials such as mono-diglycerides would orient themselves at the interface of the aqueous-lipid system. It was shown previously that pretreatment of the membranes with ethyl ether removed the carotenoids and squalene-like compound (Figure 24). Thus, it seems logical to assume that they are loosely bound and surface oriented as suggested by White et al. (1954).

Ultracentrifugal analyses of this center-cut were quickly realized (Figure 35). However, the minimum solubility of this protein fraction prevented more specific ultracentrifugal analysis of the protein. It was noted during the ultracentrifugal runs that a large part of the material sedimented out at 20-25,000 r.p.m., the sedimenting material being mucoidal in nature. When run in veronal buffer at pH 8.6, $\Gamma/2 = 0.1$ and 0.05, and in water solution, a two component system was observed with a small leading peak too diffuse to measure accurately. The sedimentation coefficients, given in Table 14, give values of 17 - 18.8 and 5.8 - 8.62 for peaks 1 and 2, respectively, depending upon the solvent employed. Attempts to electrophorese the center-cut were in vain since lipid refraction impaired accurate photography of the fraction.

However, consideration will be given to a salt fractionation scheme, presently, which was capable of producing a solution of sufficient clarity as to be observed electrophoretically.

Figure 33 represents an ultracentrifugal diagram of the sucrose fractionated center-cut after removal of the lipid portion by solvent extraction. This fraction possessed an S_{20} of 11.78 at approximately 0.70% in veronal buffer. The symmetry of the sedimenting boundary and S_{20} is very similar to that reported by Ramachandran and Whitney (1960), a value of 8.84 $\pm$ 0.73. When this fraction was analyzed ultracentrifugally at pH 12.0 (KOH buffer), the sedimentation coefficient was 11.13 S, whereas at pH 7.1 (phosphate buffer), three components of S_{20} = 32.23, 27.35 and 15.35 were observed (Table 14). This indicates the heterogeneity of this fraction. Its electrophoretic pattern (Figure 33) and electrophoretic mobility of -4.29 are identical to the values reported in Table 11 for the soluble membrane-protein at the same pH.

Lest the world around us fall, we should not be dismayed. Figures 36 and 37 show the results of freeing another membrane center-cut lipoprotein of its lipid. The fraction is no longer singly peaked and symmetrical as in Figure 33. This researcher, however, does feel that the apparent homogeneity of the fraction in Figure 33 was due largely to low concentration. By varying the concentration in an $S_{20}^{c=o}$ study, 1.5 and 1.0% soluble proteins are distinctly ultracentrifugally heterogeneous, whereas at 0.75% this feature is not quite as apparent.

Realizing this, then, we must not misinterpret the data by analysis at only one concentration. Figure 37 aptly describes the dependency of the sedimentation coefficient on the concentration of the protein. Extrapolation to zero concentration shows two components of $S_{20}^{c=o}$ of 12.25 and 8.5. The positive slope of the plot indicated molecular interaction at higher concentrations when this sample was analyzed ultracentrifugally. The lipid free center-cut contained 11.4% nitrogen and 0.63% phosphorous, which is similar to all of the soluble membrane proteins reported.

Fractions obtained by fractionation of membrane sere with a density of 1.06 g./cm.^{3} employing NaCl as the gradient, presented some interesting results. First of all, the use of NaCl (an electrolyte) definitely had an affect upon disrupting the lipid-protein complex as evidenced by (a) a considerable fat plug and (b) far less protein in the center-cut. Figure 38 and Table 15 show electrophoretic characteristics of this fraction. At pH 9.0, in borate buffer, it was necessary to lower the ionic strength of the solution in order to visually observe the electrophoretic migration, thus the correspondingly higher mobilities of -11.23 and -9.25 as compared to -5.77 and -4.80 at pH 8.6, $\Gamma/2 = 0.1$, and -4.34 and -3.42 at pH 7.1, $\Gamma/2 = 0.1$. It is debatable in Figure 38 as to whether the leading electrophoretic peak is a true component or a fat "spike." This observation is especially true at pH 8.6. The second peak (Figure 38, B) with an electrophoretic mobility of -4.80

certainly corresponds favorably to that of the major electrophoretic component in the soluble membrane-protein fractions.

Figure 39 represents the ultracentrifugal analyses of this fraction. The first frame of each trial is presented to show gross polydispersion at the indicated prespeeds. The protein is reasonably homogeneous and sediments at rates of 5.77 (pH 9.0, $\Gamma/2 = 0.05$), 5.90 (pH 8.6, $\Gamma/2 = 0.1$) and 17.53 (pH 7.1, $\Gamma/2 = 0.1$). These data show that the fraction is grossly dissimilar from the sucrose fractionated center-cut. Unfortunately, sufficient quantities of protein could not be obtained to analyze for nitrogen and/or phosphorus. The author definitely questions his own choice of an electrolyte in separating the lipoproteins, however, since the dielectric constant is altered markedly, and the opportunity for dissociation of the complex is increased.

The fat plugs obtained from both sucrose and NaCl fractionations of the membrane material were essentially all lipid material and solid at room temperature. It is believed, although not accurately known, that the lipid material was practically all HMGFraction and phospholipid, the former comprising the bulk of the lipid present.

Table 12

Electrophoretic mobilities, and nitrogen and phosphorous contents of the soluble membrane-protein of the milk fat-globule membrane

Researcher	Mobility[a] (cm^2 $volt^{-1}$ $sec.^{-1}$) Peak number: 1	2	3	Nitrogen (%)	Phosphorous (%)
		Glycine-HCl			
This study, pH 2.1	+5.78	+4.44	+1.96	10.35 - 11.45	0.63
Ramachandran and Whitney (1960) pH 1.8	+5.92	+4.09	+2.25	11.58, 11.57	0.78, 0.68
Brunner & Herald (1958), pH 2.05	+5.50	+4.16	+2.07	11.10	0.46
		Veronal			
This study, pH 8.6	-1.95	-4.33			
Ramachandran and Whitney (1960) pH 8.6	-2.81	-4.52	-5.22		
Brunner & Herald (1958), pH 8.7	-3.03	-4.23	-5.41		

[a]Calculated from initial boundary, descending limb

Table 13

Lipid composition of the pellet and supernatant from sucrose fractionation of membrane sera

Lipid[a]	Pellet		Supernatant	
	Lipid eluted from silicic acid (mg.)	Bound membrane lipid (%)	Lipid eluted from silicic acid (mg.)	Bound membrane lipid (%)
Carotenoid	0	0	1.2	0.33
Squalene[b]	0	0	3.7	1.0
Cholesteryl esters	0	0	5.4	1.5
Triglyceride	100.0	44.21	182.5	50.54
Fatty acids	10.6	4.69	19.7	5.45
Cholesterol	8.3	3.67	8.6	2.38
Diglyceride	15.6	2.34	34.1	9.44
Monoglyceride	3.3	6.90	22.0	6.09
Phospholipid	86.4	38.20	83.9	23.23
Recovered	226.2		361.1	
Charge	225.6		355.1	
% recovered	100.27		101.69	

[a]Listed in order of elution from silicic acid column

[b]Precise identification still pending

Table 14

Sedimentation-velocity coefficients (S_{20}, uncorrected) for milk fat-globule membrane protein fraction measured from corresponding sedimentation velocity patterns shown in the designated figures

Fraction	Sedimentation boundary		
	1	2	3
Figure 33A	11.78		
pH 12.0, KOH buffer[a]	11.13	7.07	
pH 7.1, phosphate buffer[a]	32.23	27.35	15.35
Figure 35			
A	17.15	5.83	
B	17.00	7.72	
C	18.80	8.62	
Figure 36			
A	19.34	15.64	
B	17.32	13.86	
C	15.70	11.91	
Figure 39			
A	5.77		
B	5.90		
C	17.53		

[a]Protein fraction from Figure 33A run in KOH and phosphate buffers

Table 15

Electrophoretic mobilities for milk fat-globule membrane protein fractions measured from the corresponding electrophoretic patterns in the designated figures

Fraction	μ (cm.^{2} volt^{-1} sec.$^{-1}$)		
	1	2	3
Figure 30			
A	-4.40[a]	-2.37	
	-4.33[b]	-1.95	
B	-4.21	-2.19	
	-4.12	-1.73	
C	+6.87	+5.66	+3.05
	+5.78	+4.33	+1.96
Figure 31			
A	-4.20		
	-3.95		
B	-4.94		
	-4.72		
C	-4.12		
	-4.04		
Figure 33			
B	-4.65		
	-4.29		
C	-4.51		
	-4.15		
Figure 38			
A	-10.73	-8.70	
	-11.23	-9.25	
B	-5.80	-4.82	
	-5.77	-4.80	
C	-4.20	-3.28	
	-4.34	-3.42	

[a], [b] Represent mobilities calculated from ascending and descending boundaries, respectively

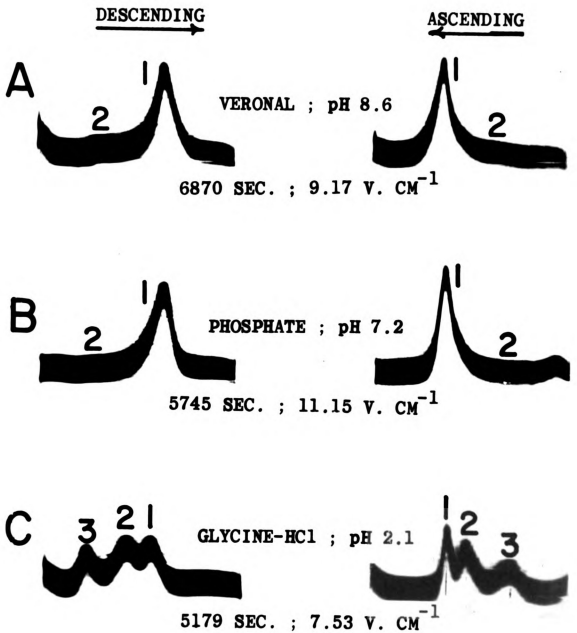


Figure 30. Electrophoretic pattern of the soluble membrane protein in (A) veronal buffer, (B) phosphate buffer and (C) glycine-HCl buffer at concentrations of 1.3, 1.0 and 1.3, respectively.

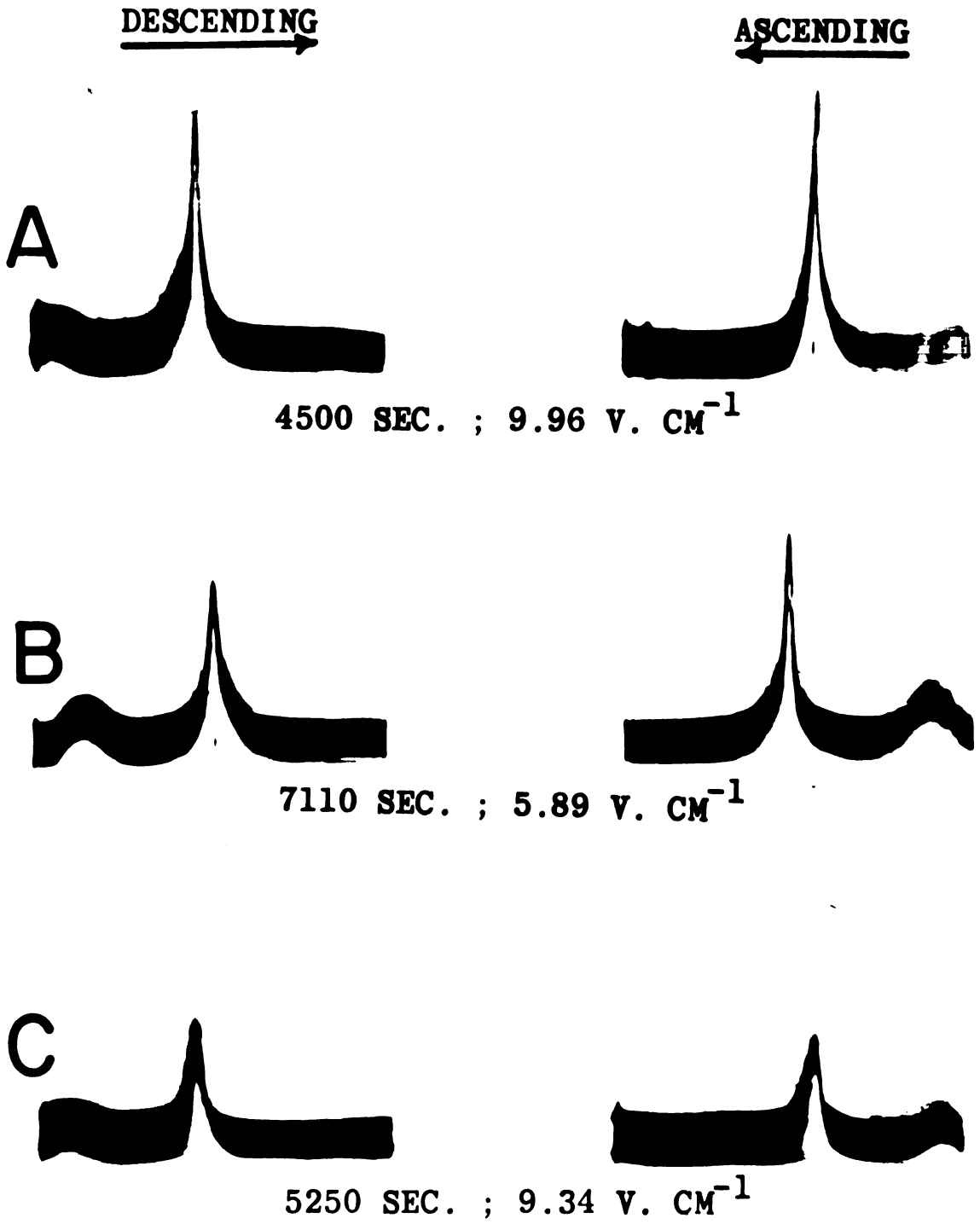


Figure 31. Electrophoretic patterns of insoluble membrane proteins solubilized with (A) basis solution, (B) 3% peracetic acid and (C) 2' thiodiethanol at concentrations of 0.60, 0.53, and 0.40% respectively. The fractions were run in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$.

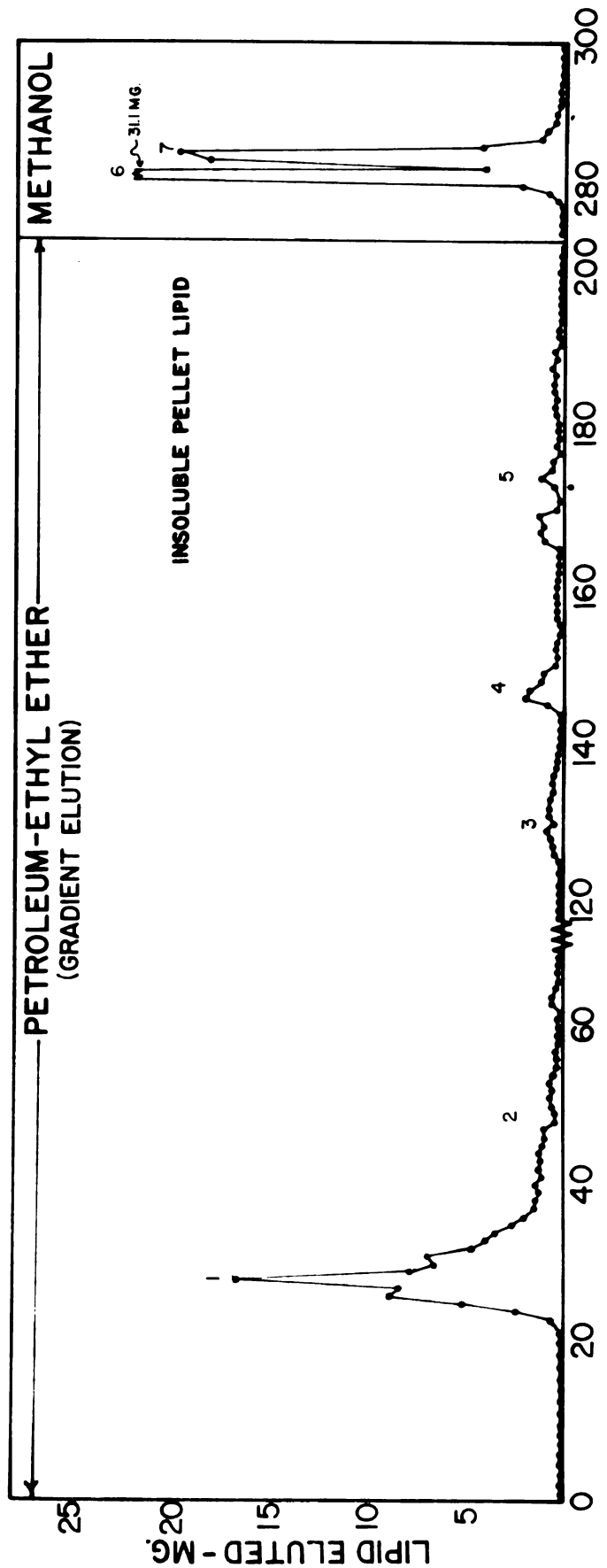


Figure 32. The separation of lipid components of the insoluble membrane pellet obtained by sucrose fractionation of membrane sera eluted from an 18 g. column of silicic acid. Lipid charge was 225.6 mg.

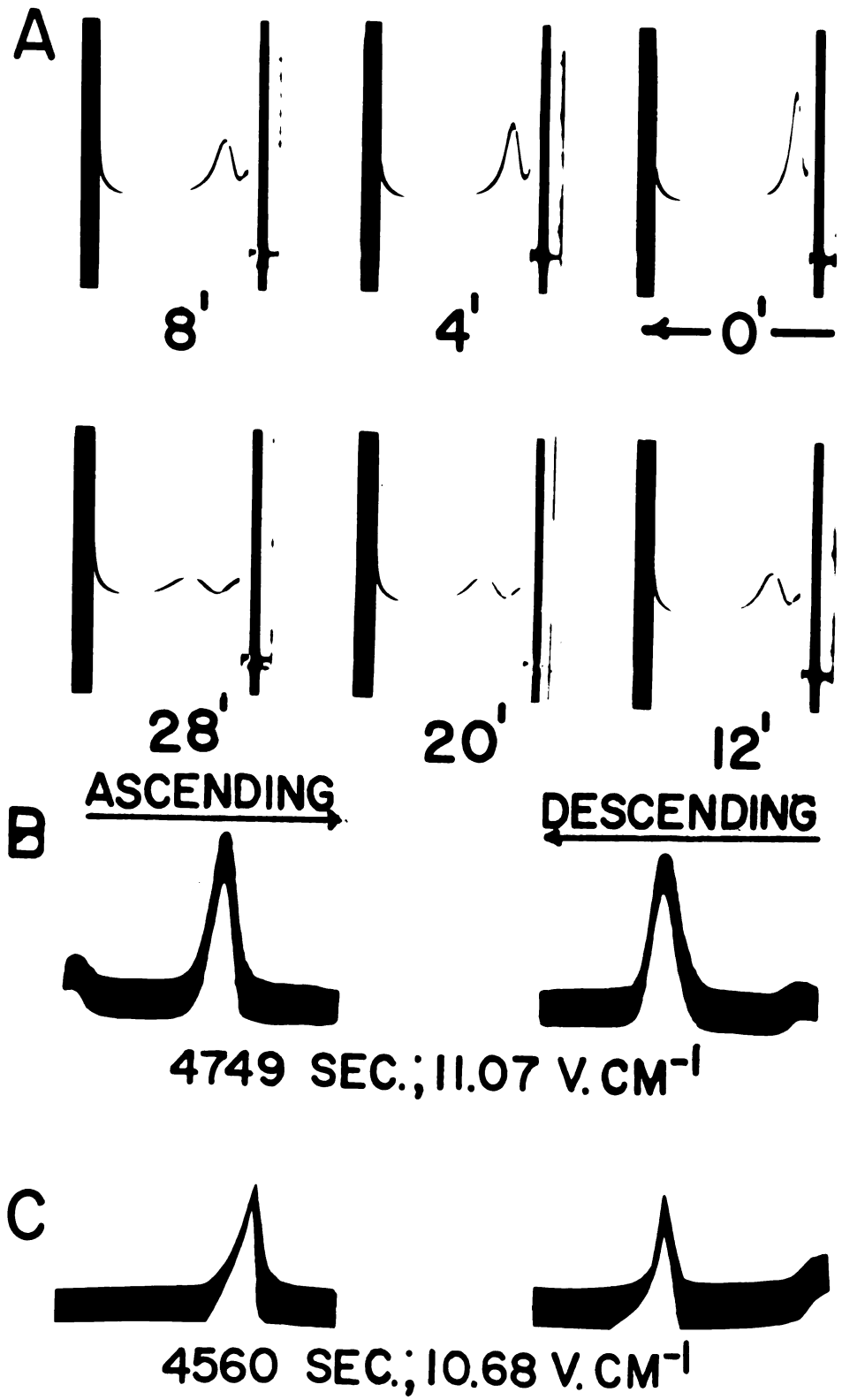


Figure 33. (A) and (B) represent ultracentrifugal and electrophoretic analyses of the lipid-free protein obtained from sucrose fractioned membrane sera. The protein concentrations for (A) and (B) were 0.70 and 1.0%, respectively. (C) represents the electrophoretic pattern of the native insoluble membrane protein at 0.5% concentration. All fractions were studied in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$.

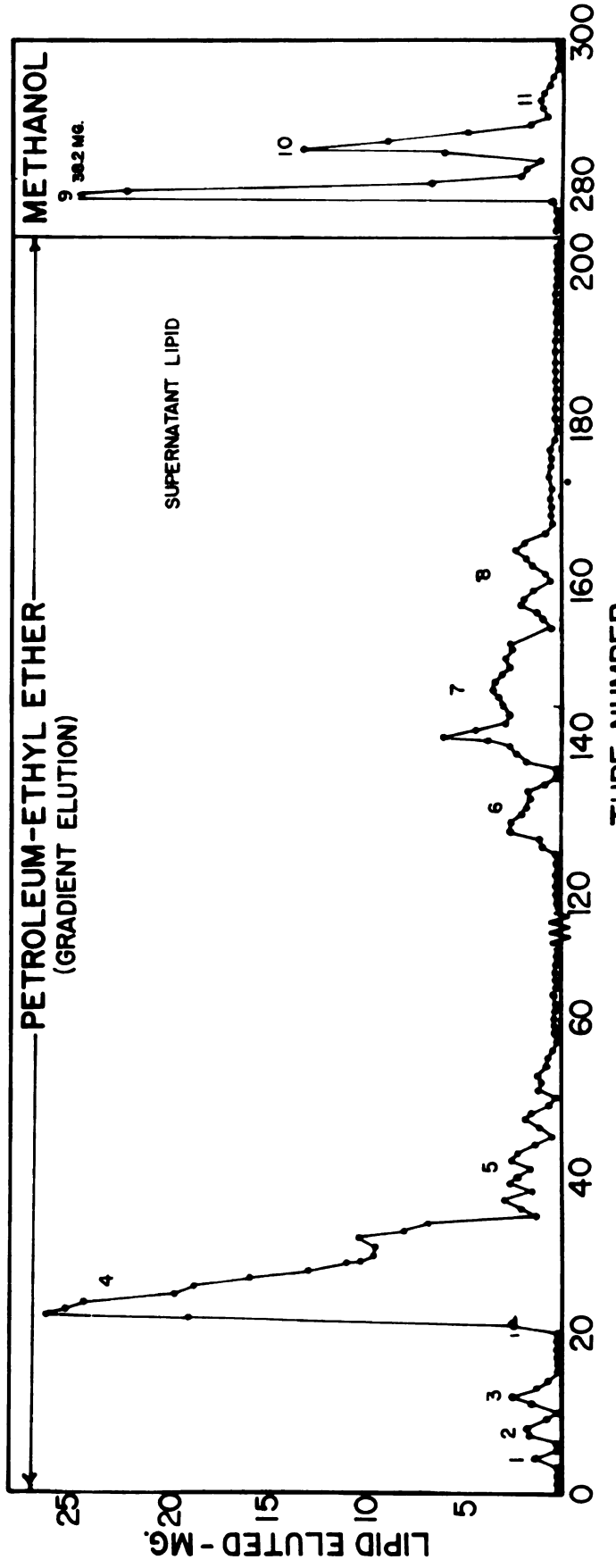


Figure 34. The separation of lipid components of the center-cut, obtained by sucrose fractionation of membrane sera, eluted from an 18 g. column of silicic acid. Lipid charge was 355.1 mg.

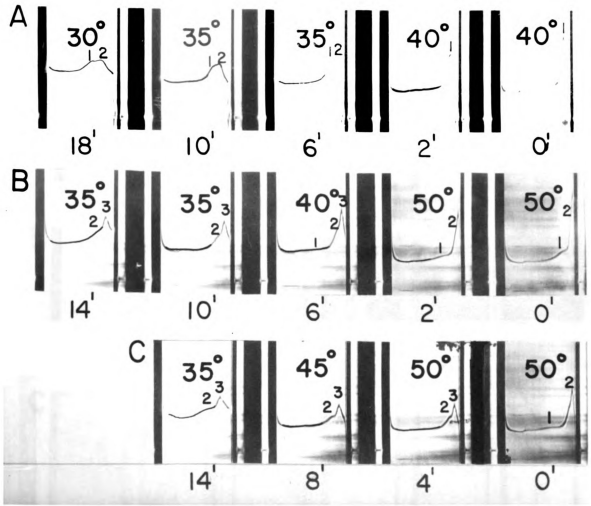


Figure 35. Ultracentrifugal diagrams of membrane lipoproteins fractionated with sodium chloride. (A), (B) and (C) represent analysis in water, veronal buffer at pH 8.6, $\Gamma/2 = 0.1$ and veronal buffer at pH 8.6, $\Gamma/2 = 0.05$ respectively.

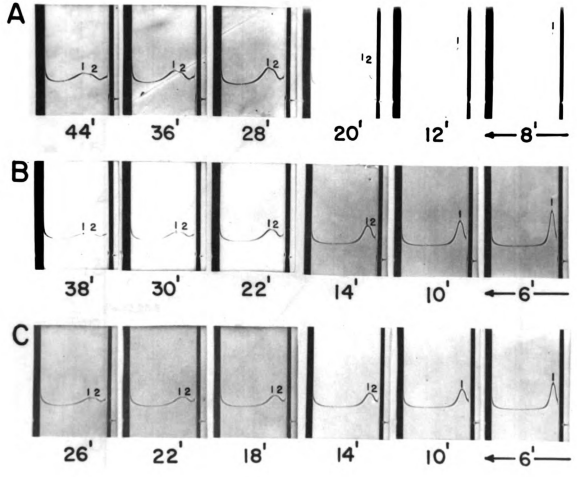


Figure 36. Ultracentrifugal diagrams lipid-free proteins obtained by ethanol-ether extraction of the sucrose fractionated lipoprotein. Protein concentrations are 1.5% for (A), 1.0% for (B), and 0.75% for (C). The buffer system was veronal at pH 8.6, $\Gamma/2 = 0.1$. The rotor was operated at 52,640 r.p.m. with the phase plate maintained at 60°.

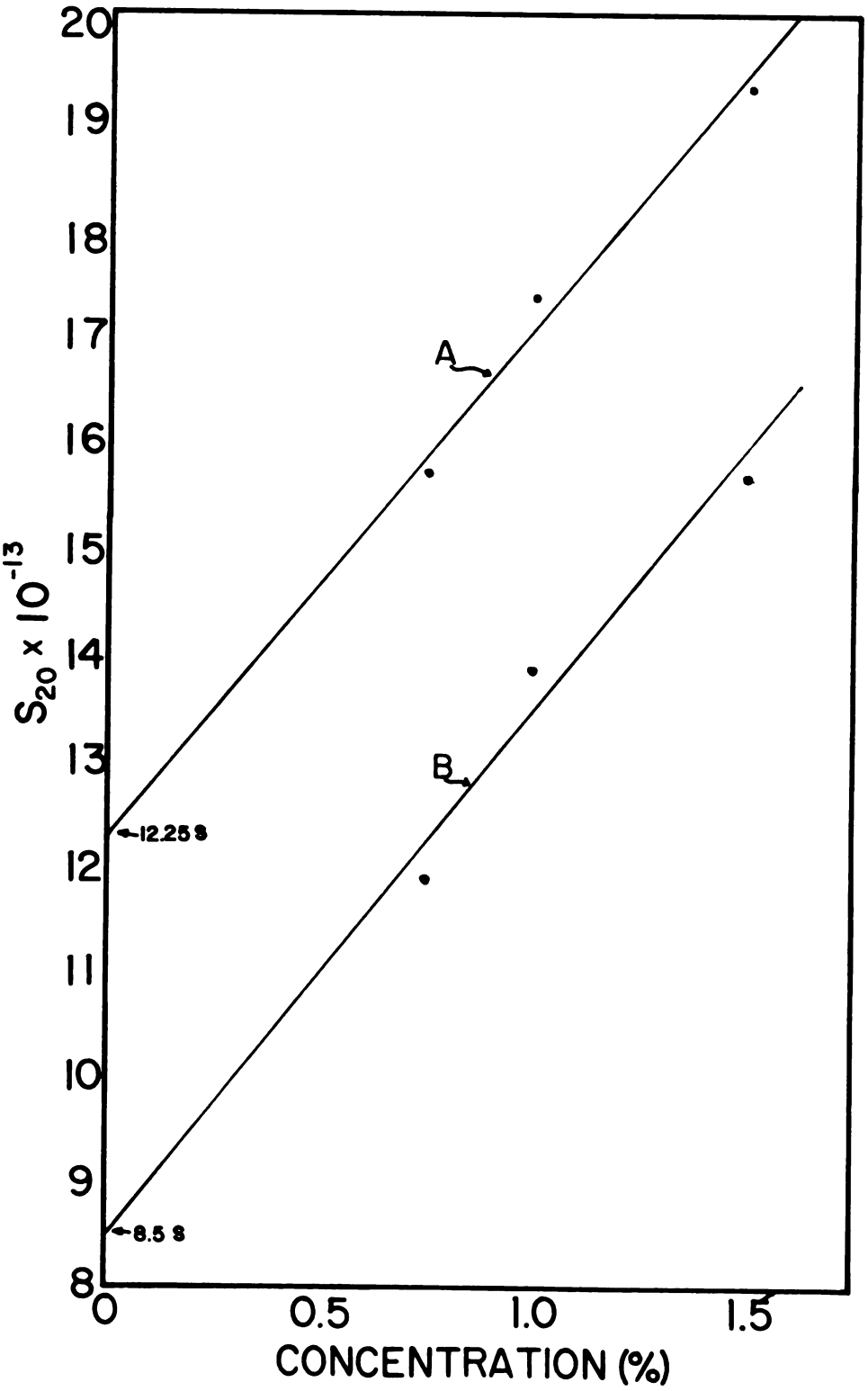


Figure 37. Extrapolation of sedimentation velocity coefficients to zero concentration for the lipid-free proteins obtained from sucrose fractionated lipoproteins.

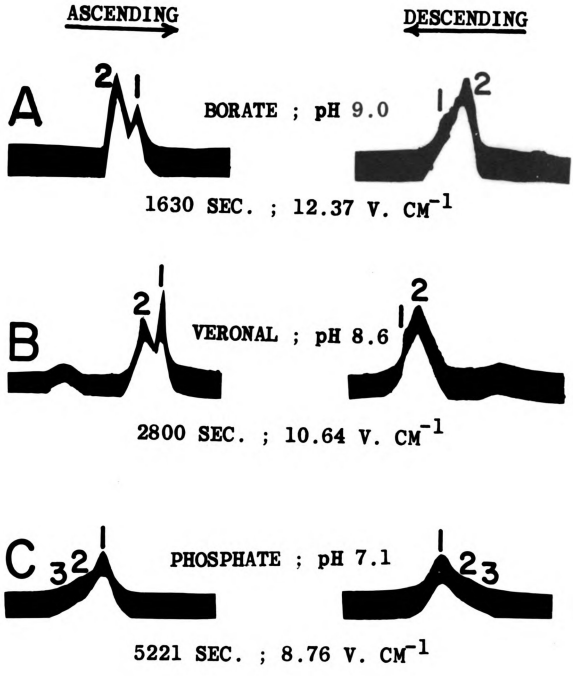


Figure 38. Electrophoretic patterns of NaCl fractionated lipoproteins at approximately 0.75% concentration in buffers indicated. (A) was run at $\Gamma/2 = 0.05$, (B) and (C) at $\Gamma/2 = 0.1$.

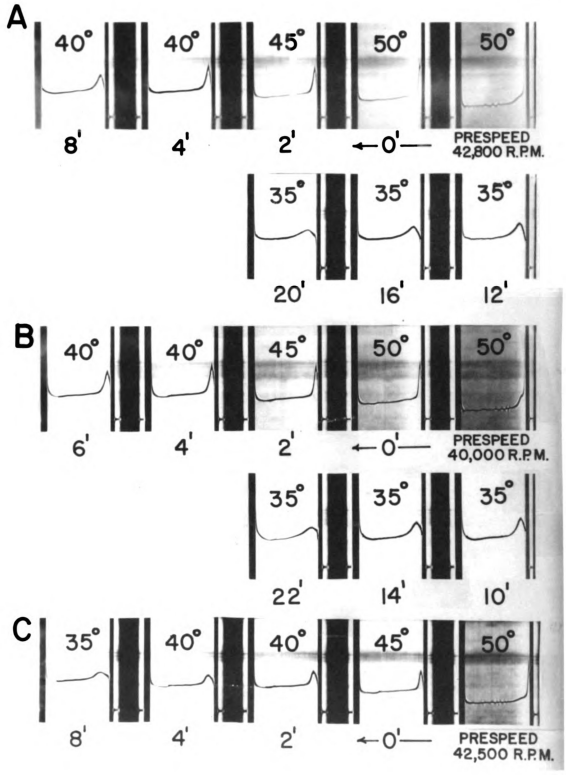


Figure 39. Ultracentrifugal diagrams of salt fractionated lipoproteins in (A) borate buffer, $\Gamma/2 = 0.05$, (B) veronal buffer, and (C) phosphate buffer at $\Gamma/2 = 0.1$.

SUMMARY AND CONCLUSIONS

High-melting Glyceride Fraction (HMGF)

The HMGFraction obtained from both the fat-globule membrane and butteroil were very similar in their physical and chemical properties; iodine number of 5. 0, saponification equivalent of 200, melting range of 50-52°C., and refractive index at 60°C. of 1. 445. The HMGF constituted 44. 48% of the bound membrane lipid, and 4. 88% of the total butteroil. The high melting range of these fractions has been attributed to the high concentrations of C_{14} (11 mole %), C_{16} (59. 6 mole %) and C_{18} saturated (16. 5 mole %) fatty acids. The low concentration of C_{18} unsaturated fatty acid residues (6. 1 mole %) as opposed to the 28. 3 mole % for butteroil is further reason for the high melting range. The values presented above are in good agreement with those reported by Jenness and Palmer (1945a) and Patton and Keeney (1958).

Silicic acid chromatography of the HMGFractions indicates that the fraction is essentially 100% triglyceride. By altering the ethyl ether concentration in the gradient elution of the lipid, the HMGF could be further fractionated.

Examination of the fatty acids present in the ethanol extracts from which the membrane HMGF was crystallized showed very few fatty acids after methanolysis. This feature suggests the first conclusion from this study:

(a) The HMGF is the major triglyceride appearing in the milk fat-globule membrane, an observation which reflects its selectivity in the membrane complex.

(b) Infrared analyses have confirmed, without question, that the HMGF is truly triglyceride, and ultraviolet and near infrared analyses show that it is somewhat unsaturated. These observations coincide with the iodine number of 5. 0.

(c) The presence of long chain fatty acids (C_{14}, C_{16}, C_{18}) is important in the union of the fat globule with the remaining portion of the membrane lipid.

Chromatography of Membrane Lipids

Membrane lipids prepared by two different schemes showed significant variations, but were consistent in the following respects: First, it was determined that the major lipids existing in the membrane are triglycerides and phospholipids. Membrane lipids prepared from cooled, pooled milk revealed a larger percentage retention of triglycerides in respect to the total membrane than did lipids from uncooled, fresh milk. The deviations between the two schemes of preparation have been attributed to the retention of protein as affected by method of preparation, scheme II retaining much more protein.

Carotenoids and a squalene-like compound comprise a part of the membrane lipid, but are loosely bound as evidenced by their complete removel after extraction with ether. Cholesterol and its esters comprise

4-9% of the membrane lipid. The occurrence of unesterified fatty acids and mono-diglycerides is of major interest. Apparently some of the mono-diglycerides exist on the membrane surface by virtue of their high surface activity, but a portion of these occur as a result of lipolysis.

By virtue of the sharp elution of the membrane triglycerides from silicic acid as opposed to butteroil, and by virtue of the solid condition of these triglycerides at room temperature, the first conclusion of this phase of the study can be drawn, namely:

(a) The HMGFraction, previously discussed, is the major triglyceride fraction contained in the milk fat-globule membrane. This is in accord with the previous statement that the ethanol supernatant from HMGF crystallizations contained very few fatty acids.

Additional conclusions drawn from this study are:

(b) The carotenoids and a squalene-like compound are loosely bound to the membrane surface. Since the squalene-like compound was obtained in only small amounts it was difficult to characterize.

(c) Triglycerides and phospholipids account for over 70% of the membrane lipid.

(d) Cholesterol and its esters exist in the surface layers of the fat-globule, and are intricately bound in the lipoprotein complex.

(e) Mono-diglycerides appear in the membrane lipid in excess of 10%. Some of these lipids are present due to their surface activity

whereas a portion of them exist as a result of lipolysis. The latter is especially true when cooled, pooled milk has been used for membrane preparation, and is evidenced by the appearance of unesterified fatty acids.

(f) The lipid distribution and yields of total membrane are affected by the method of preparation (temperature and agitation) and the age of the original milk used.

(g) Evidence is presented which suggests that the lipid-protein complex of the membrane is disrupted by lyophilization.

Soluble and Insoluble Membrane-Proteins

The total yield of membrane material ranged from 0.94 - 1.61 g./100 g. fat depending upon the nature of the milk used for membrane preparation. The quantity of membrane lipid obtained per 100 g. fat was a constant value, whereas the protein yield was a variable factor. For example, membranes obtained from uncooled milk yielded 1.61 g./100 g. fat of which 0.91 g. was protein. When cooled, pooled milk was employed for the isolation of membranes, the protein content was 0.73 g./100 g. fat or less.

Approximately 60% of the total protein was found to be soluble membrane-protein; the remaining 40% consisted of the insoluble membrane-protein fraction.

Conclusions drawn from this study are:

(a) The quantity of membrane material obtained per 100 g. of milk fat was dependent largely upon preparative procedures.

(b) The quantities of soluble and insoluble membrane-proteins were approximately equal.

(c) The insoluble membrane-protein was freed from the membrane complex as a result of the churning process.

Soluble and Minor Protein Fractions

The soluble membrane-protein was compared with the proteose-peptone, sigma-proteose, minor protein-fraction of Weinstein et al. (1951a) and whey component 5 fractions. The elemental analysis of these fractions showed that they all contained phosphorus and hexose carbohydrates, and to be high in ash. The presence of carbohydrates corresponds well with the previously reported data of Thompson and Brunner (1959c). Proteose-peptone and sigma-proteose were almost identical in nitrogen content (12.8%), whereas whey component 5 was 1% lower. The minor protein-fraction of Weinstein et al. (1951a) and the soluble membrane-protein were both low in nitrogen (10.30%), the latter fraction containing considerable quantities of fat. All of the fractions, except soluble membrane-protein, were sensitive to the action of rennin especially after heat treatment.

In veronal buffer all of the fractions studied possessed one major electrophoretic component of approximately the same mobility. In acid buffer, the skimmilk minor proteins (proteose-peptone, sigma-

proteose, and whey component 5) could easily be discerned by the appearance of a sharp electrophoretic component. However, the minor protein-fraction and soluble membrane-protein did not possess this characteristic; in the former case, the high concentration of rennin scission products probably obscured the component. Ultracentrifugally, the four major whey minor proteins possessed a major peak with an S_{20} of approximately 1. Proteose-peptone, sigma-proteose, and minor protein-fraction showed a small leading component of an S_{20} approximating 2.8. Whey component 5 possessed a leading component with an S_{20} of 5.88, which is similar to beta-lactoglobulin. The soluble membrane-protein was completely dissimilar to the skimmilk proteins showing two rapidly sedimenting components with S_{20}'s of 17.6 and 9.05.

After heating at 95°C. for 30 minutes, the soluble membrane-protein became more resolved electrophoretically in veronal buffer, and less resolved in HCl-NaCl buffer. Heating of whey component 5 did not affect general electrophoretic properties, but did lower the mobilities. Ultracentrifugally the soluble membrane-protein boundaries became more discernible and the S_{20}'s were somewhat lower, which indicated the dissociating influence of heat. The sedimentation boundary ascribed to beta lactoglobulin in whey component 5 was absent after heating, and a new boundary (S_{20} = 2.8) similar to the other skimmilk proteins appeared.

Several conclusions are to be drawn from this study:

(a) On the basis of elemental analyses, all of the proteins studied were very similar.

(b) Heated proteins were generally affected by a reduction in electrophoretic mobility, and sedimentation coefficient, thus indicating that heat alters the proteins.

(c) The minor protein-fraction of Weinstein et al. (1951a) is rich in scission products of the rennin reaction. This feature makes the bulk of the protein uncommon to normal milk except for the proteose-peptone that it contains.

(d) On the basis of ultracentrifugal analysis, the soluble membrane-protein is completely dissimilar to the skimmilk minor proteins at least in respect to molecular aggregation. Considering the source of this protein, and unless one subscribes to the "common core" theory of protein anabolism, it is extremely doubtful if the protein is similar to the minor proteins of skimmilk.

(e) Sigma-proteose is similar to proteose-peptone except that it is considerably deficient in what appears to be component 8, component 8 being at least partially soluble in 0.5 saturated $(NH_4)_2SO_4$. This similar absence of component 8 appears in the minor protein fraction which is also precipitated by $(NH_4)_2SO_4$.

(f) Whey component 5, as described by Jenness (1959), is contaminated with components 3 and 8 plus other proteins not

existing in proteose-peptone, sigma-proteose, or minor protein-fraction. The procedure of Jenness is recommended for securing the gross proteose-peptone fraction on the basis that no heat is applied during the preparation. To obtain pure components 3, 5 and 8 other separating techniques are to be recommended.

(g) All of the minor serum proteins (non-heat coagulable) and the soluble membrane-protein are recommended by this author to be termed "non heat-coagulable serum glycoproteins" or simply "serum glycoproteins."

Lipoproteins

A portion of this study was dedicated to studying the soluble membrane-protein of Brunner and Herald (1958) and a similar fraction reported by Ramachandran and Whitney (1960). Data compiled in this study suggest that the soluble membrane fractions are identical on the basis of electrophoretic examination at pH 8.6 and at approximately pH 2.0, phosphorus content (about 0.60%) and nitrogen (11%). The inhomogeneity of the fractions in the ultracentrifuge has been attributed to the amount of residual lipid remaining in the fraction following solvent extraction. Evidence has been presented previously (Herald and Brunner, 1957; and Thompson and Brunner, 1959) that the soluble membrane-protein is a glycoprotein.

The insoluble membrane-protein can be solubilized at pH 11.8 in aqueous solution, with 3% peracetic acid and with 2' thiodiethanol,

the latter two compounds being able to oxidize S-S linkages to S-H or SO_3H. The solubilized protein can be electrophoresed and the electrophoretic mobilities fell in the region of -4.0 $cm.^2$ $volt^{-1}$ $sec.^{-1}$ Low angle x-ray diffraction studies have indicated that the insoluble membrane-protein is fibrous in nature.

Differential sedimentation of membrane sera, employing 10% sucrose, offered an excellent technique for separating the membrane into (a) a fat plug, (b) center-cut and (c) an insoluble pellet. Lipid analyses of the insoluble pellet showed it to be devoid of carotenoids, cholesterol esters and a squalene-like compound. The ratio of triglyceride to phospholipid was 1:1, and the phospholipids cephalin and lecithin were in a 1:1 ratio with sphingomyelin being absent. These data induced the following conjectures: (a) Since the insoluble protein fraction was free of carotenoids, and the squalene-like compound, it was considered that these lipids are not affixed in the immediate vicinity of this protein. (b) The shift in the cephalin-lecithin ratio from approximately 1:.5 (w/w) in the original membrane lipid to 1:1 in this fraction was suggestive that these molecules are oriented with the remainder of the lipid protein complex in some specific manner.

The center-cut from sucrose fractionated membranes contained 45.64% lipid of which 50.54% of the total lipid was triglyceride and 23.23% was phospholipid. The high concentrations of carotenoids, squalene-like compound and mono-diglycerides suggested that this fraction was the true outer layer of the membrane surface since

surface active materials, such as mono-diglycerides, would orient themselves at the aqueous-lipid phase of the system. It had been shown previously that ethyl ether removed the carotenoids and squalene-like compounds which suggested that they are loosely bound, and surface oriented.

Electrophoretic observations of the sucrose center-cut were fruitless since the proteins produced optically opaque solutions. However, ultracentrifugal analyses in water and in veronal buffer showed the fraction to be two components with sedimentation coefficients of 17.0 and 7.72 at 0.1 ionic strength. After lipid removal from the fraction, one trial gave an S_{20} of 11.78 for the "homogeneous" soluble protein fraction, whereas another trial (with less lipid removed from the protein) gave a two component system with $S_{20}^{c=o}$ of 12.25 and 8.5. Electrophoretically, this soluble fraction possessed a mobility of -4.29 which was identical to other soluble protein preparations.

When sodium chloride solutions were used to fractionate membrane sera, the resulting center-cut could be observed electrophoretically, and at pH's of 7.0 - 9.0 the protein was heterogeneous. In the ultracentrifuge, the fraction appeared homogeneous in all buffers used.

Conclusions drawn from this section are as follows:

(a) The soluble membrane-protein of Brunner and Herald (1958), Ramachandran and Whitney (1960) and data on this fraction from this study show that the fractions obtained are electrophoretically

identical, and identical by elemental analyses. Ultracentrifugal variations, reflecting the state of aggregation, presumably are due to the extent of lipid removal.

(b) The insoluble membrane-protein is electrophoretically homogeneous when solubilized by different methods.

(c) The insoluble membrane-protein is fibrous in nature as determined by low angle x-ray diffraction studies.

(d) Sucrose fractionated insoluble membrane-protein contains only 8% lipid. A 1:1 ratio of triglyceride to phospholipid exists in the fraction with a similar 1:1 ratio of cephalin to lecithin (w/w). The absence of carotenoids and a squalene-like compound suggest that the protein did not come in contact with these substances.

(e) A sucrose fractionated center-cut, containing large amounts of carotenoids, the squalene-like compound, and mono-diglycerides was the true outer layer of the fat membrane.

(f) The sucrose center-cut could be analyzed ultracentrifugally, and was two component in nature.

(g) Lipid free center-cuts from sucrose fractionated membranes contain the soluble membrane-protein with identical electrophoretic properties of this fraction. The nature of the soluble membrane-protein obtained depends upon the degree of lipid removal from the protein.

(h) Sodium chloride fractions of membranes can be obtained. However, this procedure is not recommended because NaCl definitely disrupts the protein-lipid linkage yielding proteins of unknown properties.

Structure of the Fat-globule Membrane

As was intended, a major purpose of this thesis was to depict a logical structure of the milk fat-globule membrane on the basis of analytical results. But before this can be accomplished, several features of this study, and the research of others must be re-emphasized. First of all, it appears from the data of Jenness and Palmer (1945a) that the high-melting glyceride (HMGF) is radially oriented into the fat globule itself, and when butter melts a considerable amount of the HMGF is drawn into the butter plasma by the lipoprotein. From data presented in this thesis it was concluded that the HMGF is composed of long chain fatty acid, primarily. Also, from gas chromatographic analysis of ethanol supernatants from HMGF crystallizations during the preparation procedure, and from the nature of the diagram and melting range properties of the triglycerides from membrane lipids it was concluded that the HMGF constituted most, if not all, of the membrane triglyceride.

Secondly, the role of the insoluble membrane-protein in the membrane complex has never been determined. There is no doubt that after churning of cream the fraction is easily sedimented from

suspension. This indicates that in disrupting the fat-in-oil emulsion the insoluble membrane-protein did not occupy a position on the outer-most portion of the membrane. Otherwise, the fraction would be freed without the necessity of churning. Likewise, the hydrophobic nature of the protein would not be conducive to stabilizing an emulsion. In addition, the high concentration, or better still, the 1:1 ratio of tri-glyceride to phospholipid makes the fraction completely different from the soluble center-cut or the whole membrane lipid. The 1:1 ratio of cephalin to lecithin is suggestive of their specificity in the linkage of the molecule to the remainder of the lipid-protein complex. The fraction is devoid of membrane carotenoids, a squalene-like compound and cholesterol esters, which indicates that the fraction never comes in contact with these substances. Data have been contributed by Herald and Brunner (1957) tentatively classifying the insoluble membrane-protein as a pseudo-keratin. In accord with this observation, x-ray diffraction studies have shown the molecule to be fibrous.

Lastly, the fact that the soluble center-cut obtained by sucrose fractionation was richer in mono-diglycerides than the insoluble pellet suggested that the center-cut lipoprotein was the true outer layer of the membrane by virtue of the affinity of mono-diglycerides for aqueous-lipid interfaces. The abundance of carotenoids and the squalene-like compound in this fraction also suggests this to be true. White et al. (1954) suggested that carotenoids and vitamin A were loosely bound to

the membrane surface. This has been confirmed here because ethyl ether extraction of membranes removed carotenoids and the squalene-like compound.

Concluding from the data presented, the author of this thesis depicts a membrane structure very similar to that proposed by King (1955), Figure 40. The HMGF is radially oriented at the surface of the fat globule. By virtue of its long chained fatty acid residues, the HMGF's hydrophobic groups project into the triglyceride as well as into the phospholipid side chains of the lipoprotein, which constitutes the periphery of the fat globule. By nature of mutual solubility of the side chains in each other, and by virtue of van der Waals attraction forces, the rigidity of the complex is maintained. However, since the insoluble membrane-protein was fibrous in nature, contained a 1:1 ratio of triglyceride to phospholipid, was free of carotenoids and the squalene-like compound, and was released as a result of churning, the following suggestion can be made. The insoluble membrane-protein was probably intertwined among the fatty acid side chains of the HMGF and the membrane lipoprotein. The HMGFraction was an ideal substance for "cementing" the fat globule with the membrane material.

Since the lipoprotein contained a carbohydrate moiety, it is conceivable that the linkage between the protein and its lipid was due to a secondary carbohydrate-lipid linkage as well as van der Waals attraction forces.

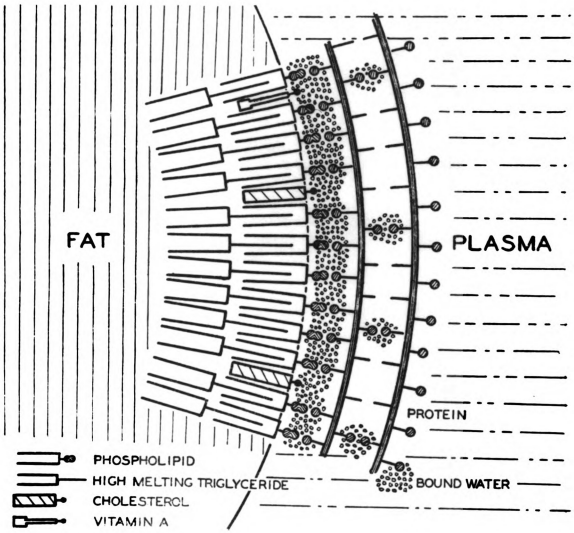


Figure 40. The structure of the milk fat-globule membrane as depicted by N. King (1955).

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