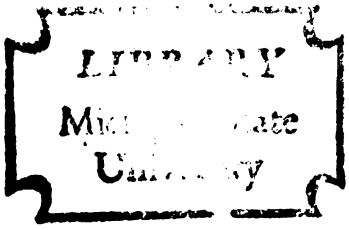
CHARACTERISTICS OF MEMBRANE LIPOPROTEIN FRACTIONS FROM COW'S MILK

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
FRED C. SWOPE
1968



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CHARACTERISTICS OF MEMBRANE LIPOPROTEIN FRACTIONS
FROM COW'S MILK

presented by

Fred C. Swope

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science

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Date July 25, 1968

ABSTRACT

CHARACTERISTICS OF MEMBRANE LIPOPROTEIN FRACTIONS FROM COW'S MILK

by Fred C. Swope

The fat globule membrane is a complex lipoprotein covering small spheres of lipids in milk. Presumably, its main function is to stabilize the lipid phase. The membrane accounts for slightly more than 1% of the total weight of the globule and consists primarily of glycerides, phospholipids, cholesterol, a structural-like protein, various enzymes and metals.

This investigation was directed toward elucidating the membrane structure more completely, using both physical and chemical methods of analyses. The fat globules were separated from other milk components and churned to fragment the membranes into various size particles. To fully utilize density and size variations, differential sedimentation was employed to separate the eroded membranes. Three pellets with approximate minimum sedimentation coefficients of 7,500S, 230S and 35S were collected and studied.

Lipid analyses showed that the 7,500S pellet contained approximately 18% total lipids while the 230S and 35S pellets increased to 30% and 55%, respectively, The phospholipids, however, accounted for 63% of the total

lipids of the 7,500S pellet but only 42% of the 35S fraction. Likewise, cholesterol decreased in concentration from the 7,500S to the 35S pellet. Lipid micelles containing surface-orientated bimodal molecules would adequately explain these observations.

The membrane protein(s) are similar in amino acid content to other milk proteins except for a lower glutamic acid and proline and a higher arginine content. When all three fractions are compared on mole basis, the 35S amino acid content exhibits more variation than the other two pellets.

The predominate characteristic feature of these lipid-extracted fractions is their insolubility in the usual aqueous systems employed to carry proteins. The three protein fractions contain carbohydrates ranging in value from 6.6% for the 7,500S to 10.1% for the 35S pellet protein.

A tentative model for the basic membrane structure has been theorized from compositional and electron photomicrographic data. A stratum-like system appears to be present in the membrane consisting of a layer of structural protein with glycolipoprotein particles anchored to it.

CHARACTERISTICS OF MEMBRANE LIPOPROTEIN FRACTIONS FROM COW'S MILK

By

Fred C. Swope

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Food Science

1968

DEDICATION

This manuscript is dedicated to the memory of my brother,

Ernest A. Swope, Jr.

August 11, 1926-October 14, 1961

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. J. R. Brunner for his valuable direction and suggestions during the course of this study, and for his assistance in the preparation of this manuscript. He is also grateful to the other members of his guidance committee, Dr. B. S. Schweigert, Dr. A. M. Pearson, Dr. A. J. Morris and Professor L. J. Bratzler.

Special thanks are due to Mr. R. J. Carroll, U.S.D.A., Eastern Utilization Research and Development Division, for kindly providing excellent electron microscopic analyses.

In sympathy the author wishes to extend an expression of gratitude to the late Dr. G. B. Wilson, Department of Botany and Plant Pathology for his timely counsel.

Lastly, the author is especially grateful to his wife, Agnes, for her understanding and encouragement throughout his graduate program.

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INTRODUCTION

The fat globule membrane is a lipid-protein complex covering the surface of fat globules in milk. Its emulsifying properties allow the lipids of milk to remain dispersed without phase separation. Upon partial or complete removal of this surface active material, the small cores of lipid coalesce to form butter. Age and addition of electron-seeking metals such as copper and iron cause objectionable flavor changes in milk which are partially attributable to the oxidative deterioration of the membrane system.

Considerable information is available on the chemical and physical properties of this heterogeneous substance. However, little is known about the physical arrangement of the chemical subunits which comprise the membrane. Even less information is available on the nature of the proteins which make up approximately 50% of it.

This study was undertaken for the purpose of investigating the membrane structure with particular emphasis on the nature of the membrane proteins. The membranes were fragmented and separated according to density by ultracentrifugation. This approach was used for several reasons. For example, if the membrane consisted of a

bimolecular leaflet as ascribed to other biological membranes, the chemical composition of the various membrane pellets should be nearly identical. The sedimentation characteristic would depend primarily on the surface area to volume ratio of each particle--assuming that all the particulates have a globular configuration. However, if the fragmented particles differed quantitatively in their composition, then each corresponding pellet would separate by density change and by size.

The fragmented membranes were separated arbitrarily into three pellets. The parameters for the pellets were defined in terms of Svedberg units. Each separation was designed to give equal distribution among the pellets. In turn, each pellet was analyzed for carbohydrates, total lipids, phospholipids, cholesterol, and amino acids. Physical data were collected on the membrane complex through electron microscopy.

A model for the structure of the fat globule membrane is proposed with aid of the data collected from this series of experiments.

REVIEW OF LITERATURE

Historical Survey of the Fat Globule Membrane

Fat exists in milk as small spherical globules ranging from 0.1 to 12 μ in diameter with the bulk of the fat in globules of 2 to 5 μ (Sommer, 1951). More than one hundred years ago Ascherson (1840) proposed the haptogen membrane theory for the material that covers these globules. He considered this material to be a condensation of albumin and other particles attached to the fat surface. At the turn of the century, Völtz (1904) stained this membrane material and observed it under the light microscope. The different staining intensities as well as varying degrees of thickness led him to the conclusion that this substance was heterogeneous. Bauer (1911), using improved staining techniques, supported the work of Völtz (1904); mainly, that the membrane stained differentially and appeared to vary in thickness.

Babcock (1885) was the first American worker to investigate this fat surface. Later, he (1889) presented evidence for small quantities of lacto-fibrin to serve as a coating for the globules. Although his conclusions were proved incorrect, his work served to stimulate a great amount of interest in the fat globule membrane which in itself was a major contribution.

In 1897 Storch, realizing the need for improved methodology for the study of fat globules, devised a method in which the fat globules were washed to remove the milk plasma. Cream was repeatedly washed with distilled water employing a cream separator. The fat was solubilized from the membrane material with organic solvents with the resulting residue being reported totally different from known milk constituents.

Völtz (1904) and Abderhalden and Völtz (1909), using a different approach, allowed the fat globules in milk to rise through columns of distilled water or salt and sugar solutions. Following hot ether extraction of the washed cream, the ether-insoluble material remaining was reported to be casein. This gravity separation technique was also used by Titus et al. (1928). They also, concluded that the protein portion of the membrane material was casein. Nevertheless, they noted that the solubility characteristics were not like casein.

Palmer and his associates (1924, 1933, 1935, 1936, 1940, 1944, 1945a, 1945b) completed a series of brilliant studies on the nature of the fat globule membrane. Unlike earlier workers who separated the membrane from the internal fat of the globules by ether extraction, Palmer and coworkers (1924-1945) churned the washed cream and recovered the membrane material from the buttermilk and the residue from the melted butter. In addition to finding phospholipids

and a high-melting triglyceride fraction, they (1933) discovered that the protein was not like any of the other known milk proteins. They (1945a) also noted that the composition of this lipoprotein was not the same in buttermilk and in butter plasma. In the former the protein/phospholipid ratio was in the range of 2.4-3.8 while the latter contained 1.0 to 2.0.

A more complete historical development of this subject is found in a treatise by King (1955).

Isolation and Amount of Membrane

One of the first serious problems to confront researchers working in this area was how to adequately isolate and concentrate the membrane material--free from milk plasma--for chemical and physical studies. Since the amount of membrane isolated depends upon such factors as globule size, temperature of milk, stage of lactation and variations in analytical procedures, little wonder that many different values have been reported (King, 1955).

In 1924, Palmer and Samuelsson were first to isolate and partially identify the globule membrane employing the churning method. Cream was repeatedly diluted with distilled water and recovered by centrifugal separation. The washed cream was churned and the buttermilk was combined with washings from the melted butter. The following year Hattori (1925) utilized a different isolation scheme. Milk

was treated with water-saturated chloroform. A partition occurred with the swollen fat globules being removed from the lower layer. After ether extraction, the proteinaceous residue was dried and called haptein. The major difference between the churning method of Palmer and the extraction procedures used by earlier investigators, particularly those employing gravity separation, was that the former method allows for the study of lipoprotein particulates of the membrane; whereas, in the latter case the lipids are usually removed by organic solvents.

Palmer and Wiese (1933), employing the washing and churning technique, found the crude membrane to range between 0.66 to 0.89 g per 100 g of fat. Jenness and Palmer (1945a) later reported the protein content to range between 0.46 to 0.86 g per 100 g of fat. More recently, the total membrane from Jersey milk has been estimated between 1.3 to 1.6 g per 100 g of fat (Chien and Richardson, 1967a; Swope and Brunner, In Review).

Mulder (1957) objected to the washing technique on grounds that constituents were removed from the surface layers of the globules. He recommended starting with uncooled fresh milk and allowing it to separate by gravity. Based on the distribution of any component between the milk, cream and skimmilk, its concentration at the fat surface can be calculated. Using this method, Mulder and Menger (1958) calculated 0.8 g of protein per

100 g of fat. Roland (1956), using an equation derived by considering the protein and fat content of milk, cream and skimmilk, estimated that 0.4 to 2.2 g of protein were present in 100 g of fat. Both methods suffer greatly from the fact that they are inaccurate and also that they estimate the loosely bound plasma proteins which are carried to the cream layer.

Unquestionably, the method of Palmer and Samuelsson (1924) for churning washed cream has been the most widely accepted procedure for the isolation and study of the membranous material. This method affords large quantities of fat globules and rapid isolation. The resulting membrane sol can be conveniently concentrated by pervaporation as suggested by Palmer and Tarassuk (1936) or by acid precipitation at pH 4.0. These concentrates are easily preserved by drying in the frozen state in a vacuum chamber.

Isotonic solutions or distilled water have generally been used as the washing media. It is particularly advantageous to employ an isotonic wash solution when labile systems such as membrane-orientated enzymes are under investigation (Dowben et al., 1967). On the other hand, membrane preparations made from these carbohydrate-rich washes are very susceptible to bacterial destruction if lengthy investigation procedures are used. In two separate studies the membrane yield was shown to increase with

isotonic washes as compared with distilled water (Erickson et al., 1964; Swope and Brunner, In Review). Although those losses have not been adequately studied, it would appear that the increased viscosity of the isotonic wash solution in part protects the membrane against erosion during centrifugal separation.

The actual effects of repeated washing on the membrane are difficult to assess from the standpoint of how many washings are necessary to remove other milk constituents without excessively eroding the membrane material. Since repeated washing is no more than a dilution process, several are needed to rid the membrane from milk plasma. Tarassuk et al. (1959) washed cream as many as fifteen times with warm distilled water without greatly affecting the emulsion properties of the membrane. This, however, does not suggest that excessive erosion is not taking place. To study the loss of membranes by repeated washing, Zittle et al. (1956) measured the activity of membrane-orientated enzymes (xanthine oxidase and alkaline phosphatase). Based on the amount of activity in the original cream, xanthine oxidase and alkaline phosphatase activities recovered in buttermilk obtained from cream washed four times were 14 and 15%, respectively. Earlier, Rimpila and Palmer (1935) found approximately one-half of the alkaline phosphatase of fresh cream removed after six washings.

Chemical Composition of the Membrane

The heterogeneity of the membrane has been recognized for many years. The first investigators, however, concerned themselves primarily with the protein component. This is not surprising since proteins were known to exhibit emulsifying properties and since fat globules formed stable emulsions. After Palmer and Samuelsson (1924) introduced the churning procedure, other membrane components were quickly discovered and investigated. Of these, the membrane lipids have been actively studied. Cholesterol and other sterols also appear to reside in the membrane as well as small quantities of heavy metals.

Although proteins represent a major part of the membrane (Thompson et al., 1961; Swope and Brunner, In Review), they are probably the least well-defined of all the components. With the exception where the membrane residue was characterized as casein (Abderhalden and Völtz, 1909; Titus et al., 1928), it has generally exhibited properties quite different from other known milk proteins. Casein and albumin have been serologically detected on washed membranes (Mulder and Menger, 1958); however, they appear to be only in minute quantities and do not deter characterization studies to any important degree (Sasaki an Koyama, 1956).

Brunner and co-workers (1953a, 1953b, 1953c), studying the membrane of both non-homogenized and

homogenized milk, showed that significant changes occur at the surface of the fat globule during processing. These studies indicated that the main protein constituents of milk plasma form a large percentage of the membrane-protein complex in homogenized milk. This would be anticipated since homogenization considerably increases the surface area to volume ratio of the fat globule; thus, creating a need for more interfacial material. When the amino acids of the native membrane were compared with those of milk plasma proteins (Brunner et al., 1953a; Hare et al., 1952), the membrane was richer in arginine, glycine and phenylalanine, but contained less aspartic acid, glutamic acid and leucine.

In subsequent experiments (Herald and Brunner, 1957; Brunner and Herald, 1958), the membrane was concentrated by salting out in 2.2 M ammonium sulfate. After lipid extraction, the proteins were dispersed in water and classified into two distinct fractions by centrifugation. The protein pellet, which represented approximately half of the membrane proteins, was reddish brown in color and quite insoluble in aqueous buffer systems. The soluble fraction showed a higher concentration of alkaline phosphatase and less xanthine oxidase than the insoluble or pellet fraction. The soluble proteins were studied electrophoretically in various buffer systems ranging from pH 2.05 to pH 8.79. One small and two large migrating

boundaries were observed. Employing strong reducing agents and detergents, the pellet was partially solubilized. Electrophoretic mobility patterns for sodium sulfide-treated samples exhibited a single, homogeneous boundary, whereas, the detergent-treated fractions were heterogeneous. These solubility characteristics indicate that part of the membrane proteins are structural in nature. On the other hand, glycoproteins have been identified in the membrane (Thompson and Brunner, 1959; Jackson et al., 1962) which implies a functional purpose.

A variety of minor proteins are known to exist in milk (Whitney, 1958). Of these, the milk enzymes have created considerable interest. The origin of these enzymes is poorly understood at present and is complicated by the fact that some may be derived from bacteria or leucocytes (McMeekin and Polis, 1949). One of the more intriguing questions left unanswered is whether they originate from the vascular system or actually represent secreted mammary gland enzymes. At present there is evidence to support both routes. Aldolase, a membrane-associated enzyme, has the same order of activity in blood as in milk (Polis and Shmukler, 1950). Bingham and Zittle (1964) found that ribonuclease A of milk is identical to pancreatic ribonuclease A which seems to suggest the possible "leakage" of this enzyme from the

blood stream. To the contrary, Folley and White (1936), studying the effects of thyroxine on milk secretion and particularly on phosphatase activity, could find no evidence to support the theory that this enzyme came from the blood serum. Consequently, they considered phosphatase a true mammary gland enzyme.

The fact that many of the milk enzymes are associated with the fat globule membrane is well documented (Brunner, 1965). Other than xanthine oxidase and alkaline phosphatase, the two major membrane enzymes, acid phosphatase (Bingham et al., 1961), aldolase (Polis and Shmukler, 1950), phosphodiesterase (Matsushita et al., 1965), lipase (Tarassuk and Frankel, 1957), DPNH-cytochrome c reductase and DPNH-diaphorase (Morton, 1953c) have been reported as constituents of the fat globule membrane. More recently Dowben et al. (1967) have identified (Na^+-K^+-Mg^{2+})-activated ATPase in the fat globule membrane, an enzyme believed to be involved in "active transport" across cell membranes.

Milk and particularly cream are rich sources of xanthine oxidase, a purine and aldehyde activating enzyme. As early as 1932, Tayama published an isolation procedure for it. Sharp and Hand (1940) recognized the presence of this enzyme on the surface of fat globules but referred to it only in terms of a flavoprotein. They noted that its presence accounted for the reddish color of buttermilk and

that upon heating to 176°F the flavin was released with a subsequent loss of this characteristic color. Its molecular weight has been estimated at 275,000 (Andrews et al., 1964) with the crystalline enzyme average ratios for protein, flavin adenine dinucleotide (FAD), molybdenum and iron of 1:2:(1.3-1.5):8, (Avis et al., 1956). Using the molybdenum, iron and FAD content as a criterion for estimating the amount of this enzyme, Swope et al. (1965), Swope and Brunner (in review) calculated that it makes up approximately 4% of the total weight of thrice-washed membranes. It is also interesting to note that the riboflavin content of human, mares', cows', sheep and goats' milks parallel the activity of xanthine oxidase found therein (Owen and Hart, 1962; Long, 1961; Rodkey and Ball, 1946). Since FAD is loosely bound and may be susceptible to enzymatic degradation, it could well be a source of riboflavin in milk.

Graham and Kay (1933) first demonstrated that phosphatase is concentrated on the fat surface of milk. This enzyme, also known as alkaline phosphomonoesterase, catalyzes the hydrolysis of organic phosphates. Morton (1953a, 1954) highly purified the enzyme and estimated that normal milk contains approximately 6 mg per liter. Of the total, 30-40% of it is adsorbed to the fat globules (Morton, 1953b). Since the enzyme is not present in true solution and can be released by treatment with butanol,

the remainder must be associated with small fat globules or lipoprotein complexes present in skimmilk. In agar-gel electrophoresis the enzyme from raw cream shows a pattern of at least three isozymes (Peereboom, 1966).

In 1924 Palmer and Samuelsson qualitatively identified phospholipids as a membrane component. Palmer and Wiese (1933) isolated and tentatively classified these lipids as lecithin, cephalin and sphingomylin. Calculated on the basis of non-dialyzable phosphorus, these three fractions accounted for approximately 19% of the total membrane weight. Later, Rimpila and Palmer (1935) reported that more than 50% of the membrane actually consisted of a non-phospholipid ether-extractable fraction. This fraction when recrystallized from ethanol had iodine numbers of 5.9-7.1, saponification numbers of 198.8-204.0 and melting points of 52-53°C (Jenness and Palmer, 1945b). They (1945b), therefore, concluded that the properties of this high-melting glyceride fraction were very similar to one found in butterfat and may actually represent interior lipids clinging to membrane phospholipids.

Mulder and associates (1957, 1958), in repeating part of these experiments found twice as much phospholipids (400-500 mg per 100 g of fat) with the surface layers of the fat globules as reported earlier (Palmer and Wiese, 1933). They also reported 36 mg of cholesterol per 100 g of fat globules of which 15% was present in the esterified

form. It is rather difficult to compare Palmer's data with those of Mulder since separation procedures were used which would account for a larger amount of surface material in the latter case.

In 1961, Thompson et al. extracted lipids from the fat globule membrane and resolved them by gradient elution from a silicic acid chromatographic column. The following lipid classes were identified as components of the membrane: carotenoids, a squalene-like compound, cholesterol esters, cholesterol, an unique triglyceride mixture similar to the high-melting triglyceride fraction, mono-diglycerides, phospholipids, and unesterified fatty acids from some preparations.

Rhodes and Lea (1958), in a detailed analysis of milk phospholipids, found no major difference between those of skimmilk, buttermilk or butter. The membrane phospholipids contained (in moles %) phosphatidyl ethanolamine 29, phosphatidyl serine 10, phosphatidyl choline 33, and sphingomyelin 19. The content of lipid-bound inositol and plasmalogens were 5 and 3 moles %, respectively. They (1958) reported that unsaturated fatty acids were present in both α' and β positions of the milk glycerophospholipids while other animal sources usually have unsaturated acids in the α' position and saturated acids in the β position.

The high-melting triglyceride fraction has now been characterized by several groups (Patton and Keeney, 1958; Thompson et al., 1959; Wolf and Dugan, 1964). Its infrared spectrum is indistinguishable from the spectra of tripalmitin and tristearin. Wolf and Dugan (1964) found that the trisaturated glyceride content of the membrane was 71.2%. Of the remaining triglycerides, 26% and 2.8% were of the GS_2U and GSU_2 types, respectively.

At least two vitamins may be concentrated at the surface of the fat globule. Skimmilk appears to be significantly higher in vitamin A per g of fat and also in the globule surface to volume ratio than whole milk (White et al., 1954). But the work of Kon et al. (1944) suggests that vitamin A is actually in true solution in the fat rather than located at the surface. Erickson et al. (1964) reported that tocopherol concentration was at least three times higher in the lipids of the fat globule membrane than inside the fat globule.

Spectrographic analysis of the membrane proteins showed that numerous metals are present (Herald et al., 1957). However, to quantitate these metals with a reasonable degree of precision is indeed difficult. The partition ratio of metals found in milk versus cream indicates that molybdenum, copper and iron are selectively located in the membrane (Swope and Brunner, In Review).

Of the metals associated with membrane, copper has been studied intensively (King et al., 1959; Koppejan and Mulder, 1953; Davies, 1933). Its role as a catalytic

agent in oxidative deterioration of phospholipids is well substantiated (Swanson and Sommer, 1940; King and Dunkley, 1959; Tarassuk and Koops, 1960; Smith and Dunkley, 1961). Approximately one-quarter of the natural copper of milk is bound to the surface of fat globules, probably as a copper-protein complex (King et al., 1959). Consequently, this makes the membrane the highest copper-containing protein thus far identified in milk (Samuelsson, 1966).

Physical Properties of Lipoprotein Complexes

The theoretical interpretation of the molecular structure of membranes in general comes from chemical analysis, different physio-chemical properties of living cells, x-ray diffraction of multimembranous structures, electron microscopy and particularly the studies of monomolecular films (DeRobertis et al., 1965). By integrating some of these observations, Danielli and Davson (1934) proposed that membranes are composed of a lipid layer with protein adhering to both interfaces. The lipid layer is bimolecular with the polar groups extending outwardly while the nonpolar groups are adjacent to each other. In recent years detailed examinations of membranes have resulted in a more definitive explanation of this lipoprotein interaction. All membranes have large amounts of an amphipathic molecule, usually phospholipids. These bimodal molecules will aggregate in water to form micelles which will interact with proteins (Green and Tzagoloff,

1966). Lucy and Glauert (1964), studying the structure and assembly of macromolecular lipid complexes made from various mixtures of lecithin, cholesterol and saponin, postulated that biological membranes have a globular configuration in which phospholipid micromicelles form the basic structure of the membrane. According to this model, protein molecules are located around the repeating phospholipid micelle. Experiments with model systems indicate that these protein-lipid complexes are held together by electrostatic forces, hydrogen bonding and in some cases probably by hydrophobic bonds (Cornwell and Horrocks, 1964; Payens, 1959; Eley and Hedge, 1956). These interactions and their relation to the stability of concentrated milk has been reviewed by Brunner (1962).

Several hypotheses have been proposed for the structure of the fat globule membrane. King (1955) visualized a monolayer of radially orientated phospholipid and cholesterol molecules anchored in the fat phase with their hydrophilic ends pointed toward a perpendicular layer of protein. The protein is situated in such a manner that its hydrophilic groups are electrostatically bound to the phospholipids on one side and hydrated in the aqueous phase on the other. In this scheme most of the hydrophobic side chains of the proteins are concealed by structural folding. On the other hand, Morton (1954) favored the view that the fat globules in milk are

surrounded by a continuous protein membrane to which microsomal lipoprotein complexes are adsorbed. The microsomal concept (Bailie and Morton, 1958a, 1958b) was based on the fact that discrete lipoprotein particles with similar enzymatic activities were found in mammary gland tissue and on the fat globule surface. More recently Dowben et al. (1967) have theorized that the globule membrane is a true biological membrane. Evidence to support this hypothesis is found in the ability of fat globule membrane antisera to agglutinate and hemolyze bovine erthrocytes; the permeability of the membrane to potassium; the location of the "unit" membrane structure; and detection of "microsomal" enzymes.

There is ample evidence that the membrane of fat globules is a heterogeneous combination of lipids and proteins. In 1961 Alexander and Lusena suspended material obtained by freezing washed cream in a 2% sodium desoxycholate (DOC) solution. The membrane suspension was sedimented into five fractions by differential centrifugation. These different fractions were distinguishable by sedimentation behavior, enzyme content and total lipid and phospholipid content. In a related but more thorough study, Hayashi and Smith (1965) selectively solubilized the intact fat globule with sodium desoxycholate. Water-soluble lipoprotein particles accounting for 45% of the total membrane weight were released. Chemical analysis

showed that the lipids and proteins were present in approximatley equal amounts and that 76% of the lipids was phospholipids. Comparison of these DOC-released particles and those obtained from untreated preparations of membranes indicated that the DOC treatment did not significantly alter the physical structure of the complexes. Ultracentrifugal data showed that the particles were physically heterogeneous (Hayashi et al., 1965). It was therefore concluded that the membrane consisted of two types of lipid-protein complexes. These complexes are an insoluble lipoprotein which borders the triglyceride core and water-soluble lipoprotein particles which are adsorbed to it. Chien and Richardson (1967a, 1967b) studied the gross composition of the membrane by agitating washed fat globules in distilled water rather than using a solubilizing agent. The desorbed particles as well as the interfacial material released from phase inversion were also classified by sedimentation characteristics and chemical analysis. The data in general support the conclusions of Hayashi and Smith (1965) that the globule membrane is, indeed, a complex lipoprotein system comprised of several particulates which differ quantitatively in both lipids and proteins. Morton (1954) has estimated these lipoprotein fragments to comprise 5% by weight the total milk protein and range in size from less than 30mμ to 200mμ.

Upon separation of milk into cream and skimmilk a distribution of phospholipids occurs with approximately 40% of the lipid phosphorus remaining with the skimmilk (Perlman, 1935). These phosphorus compounds are presumably derived from small unseparated fat globules and lipoproteins macromicelles which may or may not originate from fat globules. The migration of loosely-bound lipid complexes from the fat globule is a common occurrence when processing treatments such as agitation, heating or homogenization are employed. For instance, Greenbank and Pallansch (1961) calculated that 29.5% of membrane phosphorus migrates to the aqueous phase upon stirring fat globules for one minute at 20°C. Heating of milk causes about 20% of the phospholipids to be desorbed from the globule surface (Koops and Tarassuk, 1959).

Both the membrane lipoprotein and other lipoproteins prepared from unwashed cream show alkaline phosphatase activity (Sasaki and Koyama, 1959). Zittle et al. (1956) suggested that alkaline phosphatase occurs in skimmilk primarily in the same complexes as it does in cream but that xanthine oxidase may occur in smaller and less easily sedimented units in the skimmilk. Within the last several years Patton et al. (1964, 1965) have discovered a lipogenic agent in milk which has the capacity for glyceride biosynthesis. This material has physical and chemical similarities to the fat globule complexes but its exact relationship is unclear since the biosynthetic activity appears to be confined almost entirely to milk plasma.

The possibility of a lipid-casein specie has been raised by Cerbulis and Zittle (1965). They stated that acid precipitated casein contains 4-7% of complex lipids which can be differentially extracted with organic solvents. Morton (1953a) made a similar observation when acids or organic agents were used to remove casein from milk. However, he considered this material nothing more than entrapped and adsorbed lipoproteins of milk.

From the above discussion it is clear that lipoprotein micelles are easily adsorbed and desorbed from the fat globules. Whether these membrane-associated particles represent all lipoproteins of milk cannot be answered with certainty at this time. From enzymatic and compositional studies it appears that these particles may be similar in certain aspects while different in others. The data presented reject the concept of a well organized membrane structure for the fat globule. Perhaps in places an intact "unit" membrane does exist. By and large, however, lipoprotein macromicelles surround the fat globule. This material might be structurally the subunits of membranes aggregated in a random fashion.

Comparison of Several Membrane Systems

The complexity of membranes is such that a comparative analysis is very elementary at best. Their molecular interpretation has been based primarily on differential staining in conjunction with electron microscopy. Various

heavy metal stains have been employed for this purpose. Osmium tetroxide, which appears to be deposited at polar interfaces, has been used extensively to show the linear relationship of proteins in membranes. For contrast, tissue is fixed with formalin dichromate for electron staining of phospholipids. Isolation of the actual membrane for chemical analysis, however, is a very formidable task. It is exceedingly difficult to concentrate these structures free from other cellular debris, and for this reason the precise characterization of chemical components and enzymatic activities is questionable.

Most information on the chemical composition of membranes comes from the study of erythrocyte ghosts (Parpart and Ballentine, 1952). After hemolysis of the red blood cell a thin membrane (approximately 1% of the red cell weight) is left which is composed of lipids and proteins. The major protein component is fibrous and has a high molecular weight. Most of the lipids are phospholipids with cephalin representing nearly three quarters of them. These lipids have different degrees of binding and have been divided into classes by action of solvents. It has long been recognized that strongly acidic groups are responsible for the net negative charge of the erythrocyte possibly originating from phospholipids. Recent evidence indicates that the electrokinetic behavior may come from another source (Cook *et al.*, 1961). They showed

that a decrease in erythrocyte charge occurred after treatment with crystalline neuraminidase which released an acetylated neuraminic acid and reduced the charge approximately 80%.

In general, plasma membranes have not been adequately characterized due to limitations in the isolation procedures. Nevertheless, a dominant feature of these membranes is a tripartite structure ranging in thickness from 75-100 Å (DeRobertis et al., 1965). Emmelot et al. (1964), studying the chemical and enzymatic composition of plasma membranes isolated from rat liver, found for the most part, those components which appear to exemplify all membranes. They stated that per 100 molecules of plasma membrane phosphorus about 50 molecules of choline, 40 molecules of cholesterol, 17 molecules of hexose, 16 molecules of hexosamine, 9 molecules of sialic acid and 3,000 molecules of amino acids are present. Membrane enzymes, such as phosphatases and ATPase, were particularly evident. Using neuraminidase, Weiss (1963) showed that this enzyme produced significant weakening of cell surfaces. It appears, that here too, a glyco- or mucoprotein is a component of plasma membranes. This mucoid substance may effect cell aggregation and determine their antigenic properties.

After examining fat globules with the electron microscope, Schwarz (1947) reported that this membrane also consisted of three layers. The inner layer was thin

but optically dense; the middle layer appeared to contain a ring of phospholipid droplets; and the outer layer was thick and presumably made of protein. Knoop et al. (1958) estimated this structure to range from 5-10 mμ in actual thickness. As with other types of membranes, the globule membrane is negatively charged having an isoelectric point at pH 4.3 (Jack and Dahle, 1937). An increase in globular mobility occurs as the number of washings of the cream is increased (Tjepkema and Richardson, 1966). This is probably due to erosion of material from the polar ends of phospholipids.

One of the more interesting facets of the fat globule membrane is the phenomenon of creaming. This subject has been treated in detail by Dunkley and Sommer (1944). They concluded that globular clustering is an agglutination mechanism common to both fat globules and bacteria with a globulin being essential in each case. The removal of this material or heat denaturation of it will hinder this clustering effect. Payens et al. (1965) presented evidence to support this hypothesis in the case of fat globules. They suggested that agglutination is brought about by an euglobulin bridge between the individual globules.

Admittedly, the available data for membrane comparison are fragmentary and should be used only as a guideline. Much additional work is required to clarify this

area of research. For instance, the glyceride compounds of the fat globule membrane may not be an actual part of the true membrane system. Vasić and deMan (1966a, 1966b) suggested that the high-melting glycerides are in reality an artifact resulting from crystallization in the peripheral layer of the globule and should not be considered a true constituent of the membrane.

It is, indeed, interesting to compare certain aspects of these membrane systems. Several enzymes appear to be common to all--the esterases and ATPases. They are also characterized by high phospholipid and cholesterol content. The proteins are essentially of a high molecular weight and aggregate into large complexes which are insoluble with normal treatment. It is apparent that a carbohydrate-containing material is an integral part of these systems and probably determines the agglutinating and antigentic properties of the membrane surfaces and in part regulate their charge.

On the other hand, many dissimilarities exist in these structures. The fat globule membrane is considerably thicker and contains many loosely-bound lipoprotein complexes not common to all membranes. Other subtle differences are detectable and probably reflect specialized functions. For example, membrane ghosts from Streptococcus faecalis protoplast contain a considerable amount of a lipid resembling glycerides which is not normally

found in other biological membranes (Ibbott and Abrams, 1964). In addition, the skeletal muscle cell membrane has a very low lipid to protein ratio (approximately 1:4) as compared with other membranes (Kono and Colowick, 1961). Emmelot et al. (1964) reported that the enzymes esterase, glucose 6-phosphatase and NADH-cytochrome c reductase are 4-9 times more active in microsomal than plasma membranes preparations. This observation is particularly noteworthy since there is continuity between these two membrane systems at certain sites. Novikoff et al. (1953) have also demonstrated marked chemical and enzymatic heterogeneity among microsomal as well as mitochrondrial fractions. Thus, the chemical and physical differences serve to place certain restrictions on any definitive interpretation of membranes at this time.

Origin of the Fat Globule Membrane

A considerable effort has been put forth to learn the origin of the fat globule. The technique of radioactive labeling has elucidated several of the synthetic pathways. Presently, the most obscure area left for investigation is the transportation or movement of these materials into the mammary gland and their organization and subsequent secretion.

The milk fat lipids are primarily derived from the lipids of the β-lipoproteins (Glascock et al., 1964, 1966) while the glycerol for these lipids appears to originate

from blood glucose (Wood et al., 1958; Luick, 1961). Once these materials are made available to the cell they are rearranged in various ways by intracellular enzymes (McCarthy et al., 1965). The phospholipids of blood, according to Graham et al. (1936), do not account for any appreciable quantity of phosphorus compounds of milk. Such compounds are derived mostly from inorganic phosphates of blood plasma.

Hayashi and Smith (1965) have raised the possibility of blood lipoproteins making up in part the loosely-adsorbed material that surrounds the fat globule. This suggestion is plausible since certain milk proteins do, indeed, enter the milk performed from the blood stream (Coulson and Stevens, 1950; Polis et al., 1950; Larson and Gillespie, 1957). Larson (1965) reported that approximately 10% of milk proteins are blood proteins while the remainder are synthesized in the mammary gland. Using fluorescent antibody gamma globulin, Dixon et al. (1961) found that the acinar epithelieum allows selective passage of certain blood proteins while restricting others. These proteins pass through the cytoplasm and are discharged into the acinar lumen by an undisclosed process. In contrast, proteins which are synthesized by the mammary tissue seem to be packaged and released to the lumen by discrete action of the Golgi apparatus.

However, little evidence is presently available to support the view that blood lipoproteins provide any sizable amount of the material coating the fat globule. Von Muralt et al. (1964), using antigenic properties to determine the relationship of milk and blood proteins of human milk, reported that at least 13 to 18 proteins correspond to known blood serum proteins but only traces of blood lipoproteins were present.

The mechanism by which fat droplets are formed in the secreting cell is nothing more than speculation at present. Bargmann and Knoop (1959) and Bargmann et al. (1961) have shown certain events which appear to be sequential during the secretion process. Formation of lipid droplets occurs in the basal region of the secreting cell, probably in vacuoles of the ergastoplasm. Although the regulation of globular growth is completely obscure, a limiting membranous interface is evident during globular intracytoplasmic movement. At the apical area of the cell the fat globule protrudes into the lumen of the alveolus and becomes constricted off carrying with it additional cellular material. With this scheme in mind it is possible to designate at least two membranous layers--one originating from the interior of the cell and tightly bound and another capping the globule upon release from the cell.

There is ample evidence to support "selective leakage" or perhaps aprocine secretion for fat globules. Many cellular components have been identified in the membrane while others are conspicuously absent. For example, mitochondrial enzymes are missing (Morton, 1954). The presence of small amounts of RNA have been detected (Swope and Brunner, 1965) and would seem to suggest that the secreting cell is not losing to any great extent its machinery for synthesis and that the material being lost can readily be resynthesized. On the other hand, many secreting cells are sloughed off during the secreting process with the subsequent release of autolyzing enzymes and cell fragments (Paape and Tucker, 1966). This, indeed, makes it exceedingly difficult to ascertain the true nature of the fat globule membrane. Although such cellular degradation should not be viewed as the major route of secretion, it must be taken into consideration for the interpretation of the results of the secretion phenomenon.

EXPERIMENTAL

Apparatus and Equipment

The milk used in this study was collected in stainless steel cans and separated with a DeLavel disc-type separator (Model 9). Low-speed centrifugations were performed with the International Model V, size 2 centrifuge. Intermediate-speed separations were performed with a Sorvall RC2-B refrigerated centrifuge, using a type SS-34 rotor. The Beckman Model L-2 preparative ultracentrifuge, equipped with a type 30 fixed-angle rotor, was used for high-speed runs.

Cream was stored in Erlenmeyer flasks and churned on an Eberbach rotary shaker.

For weighings, a top-loading, direct reading, Mettler type K-7, a Mettler type H-16, and a Sartorius series 2400 balances were used.

Amino acid analyses were performed with a Beckman/Spinco Model 120C amino acid analyzer.

Membrane preparations were dried from the frozen state by a laboratory-constructed lyophilizer. Final drying for preparations as well as chemicals was accomplished in a temperature-controlled Cenco vacuum oven in which the vacuum was obtained with a Cenco Pressovac-4 pump.

A Beckman DK-2A ratio recording spectrophotometer, equipped with quartz cells, was used for all colorimetric determinations.

A laboratory-constructed Plexiglass electrophoretic cell was employed for starch-urea electrophoresis with a Heathkit Model IP-32 supplying the power. Excess stain was removed with an electrolytic destainer.

Chemicals and Materials

The principal chemicals used in this research with the name of the suppliers are listed below.

Sigma 121 (tris-hydroxymethyl aminomethane) was used as a primary standard and obtained from the Sigma Chemical Company. Phenol was purchased from Mallinckrodt. Acetylacetone was acquired from Fisher Scientific Company and redistilled before use. The p-dimethylaminobenzaldehyde and 2-thiobarbituric acid were purchased from Eastman Organic Chemicals and recrystallized. Specially prepared hydrolyzed starch was obtained from Connaught Medical Research Laboratories.

Mannose was purchased from Fisher Scientific Company and galactose from Pfanstiehl Laboratories, Inc. (Germany). Glucosamine hydrochloride, galactosamine and penicillin "G" were acquired from Nutritional Biochemicals Corporation, while N-acetyl neuraminic acid, tryptophan, streptomycin sulfate and the amino acid standards were

obtained from Calbiochem. Applied Science Laboratories, Inc. was the supplier of cholesterol.

All other chemicals used in this research were of reagent grade.

Chemical Methods

Nitrogen

A micro-Kjeldahl apparatus was used for the nitrogen analysis. The digestion mixture consisted of 5.0 g $CuSO_4 \cdot 5H_2O$ and 5.0 g of SeO_2 in 500 ml of concentrated H_2SO_4. Ten to 15 mg of dried protein in duplicate for each sample were digested with 4 ml of the digestion mixture over a gas flame for one hr. After cooling, 1 ml of 30% H_2O_2 was added to each flask and digestion continued for an additional hr. Finally, the digestion flasks were rinsed with 10 ml of deionized water. Each digestion mixture was neutralized with approximately 25 ml of a 40% NaOH solution. The ammonia was distilled into 15 ml of a 4% boric acid solution to which 3 drops of indicator had been added. The indicator consisted of 400 mg bromocresol green and 40 ml of methyl red in 100 ml of 95% ethanol. The distillation was continued until a volume of 50 ml of the solution was present in the receiving flask. The ammonia-borate complex was titrated with 0.020 N HCl.

The average recovery of a tryptophan standard was 97.4%.

Phosphorus

Phosphorus was determined by modifying a method described by Sumner (1944). All the analyses were performed in duplicate. For membrane protein, 10 to 16 mg were digested with 2.2 ml of 50% H_2SO_4 in test tubes. The digestion was carried out on a sand bath heated by an electric heater at a temperature of 160 to 170°C until the protein was thoroughly charred. After cooling, 8 drops of 30% H_2O_2 were added to each test tube and heating resumed for 15 min. This step was repeated until the solutions were colorless. It is important to drive off all the H_2O_2 before continuing with the determination. After final cooling, the digestion mixtures were transferred to 25 ml volumetric flasks. Five ml of a 6.6% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution and enough water were added to give a volume of approximately 15 ml. Then, 4 ml of a solution of 5 g $FeSO_4\cdot(H_2O)_7$ in 50 ml of water plus 1 ml of 7.5 N sulfuric acid were added and mixed. The ferrous sulfate was prepared immediately before use. The volumetric flasks were made up to volume with deionized water and mixed. After standing for 30 min to allow complete color formation, the transmittance (%) of the solutions were read at 660 mμ with a spectrophotometer.

For lipid phosphorus, aliquots of chloroform-methanol extracts (4 to 7 mg of lipid) were evaporated to dryness in a water bath. The digestion and color formation were

carried out in the same manner as described previously except that 50 ml volumetric flasks were used in place of the smaller ones.

Ten ml of a stock solution containing 1.3609 g of KH_2PO_4 dissolved in 1000 ml were diluted to 100 ml (0.031 mg/ml). A standard curve covering the range of 0.0 to 0.28 mg of phosphorus was prepared.

Hexose

The hexose analyses were performed by the method reported by Dubois et al. (1956).

Two to 4 mg of protein were placed in a test tube and 3 ml of deionized water were added. An equal volume of 5% phenol solution was added and mixed. Finally, 15 ml of concentrated sulfuric acid was added rapidly. As the acid entered, the test tube was rotated with a Mini-Shaker to obtain maximum mixing and heat production. After standing for 10 min, the tubes were again stirred and then placed for 20 to 30 min in a water bath at 25 to 30° C. The addition of acid and the stirring of the reaction mixture in the prescribed manner are necessary for reproductibility in this test. The transmittance (%) was read at 490 mμ with a spectrophotometer.

A standard curve was constructed covering the range of 0.0 to 200 μg galactose-mannose (1:1 w/w) per 3 ml of water.

Hexosamine

The hexosamine content of the various protein fractions was analyzed according to the procedure described by Johansen et al. (1960).

Samples weighing 1 to 2 mg were directly placed into 5 ml ampoules to which 1 ml of 4N HCl was added. The samples were frozen in a dry ice-ethanol bath, evacuated, refrozen and sealed under vacuum. Standard glucosamine-galactosamine solutions (25 µg) were treated in the same manner as the samples to serve as recovery standards. Blanks containing all the reagents were treated identically to the samples and served as controls.

Hydrolysis of the protein took 6 hr at 100° C in a hot air oven. After cooling, the hydrolyzed samples were transferred to distillation flasks. The empty ampoules were rinsed with 1 ml of 4N NaOH, followed by two 1 ml rinsings with deionized water.

One milliliter of freshly distilled acetylacetone was added to 25 ml of M Na_2CO_3 solution plus 20 ml of water. The pH was adjusted to 9.8 and the volume was made up to 50 ml. This solution was used within 30 min. Ehrlich's reagent was prepared by dissolving 2 g of recrystallized p-dimethylaminobenzaldehyde (Rondle and Morgan, 1955) in absolute ethanol containing 3.5% of concentrated HCl. This solution was diluted to a final volume of 250 ml.

Five and five-tenths milliliters of the acetylactone reagent were added to each flask containing the hydrolyzed sample. The final pH of this solution must be maintained between 9.5 and 10.0. The flasks were tightly stoppered and heated in a boiling water bath for 20 min. Then, each cooled flask was heated with a Bunsen burner until 2.5 ml of the distillate containing the steam-volatile chromogen was condensed in 7.5 ml of Ehrlich's reagent. The volumetric flasks were stoppered and mixed. After 30 min, the transmittance (%) of the solution was read with a spectrophotometer at a wave-length of 548 mμ.

Equal amounts of glucosamine and galactosamine were combined and used to construct a standard curve. The carbohydrate concentration ranged from 0.0 to 50 μg per 3 ml.

Sialic Acid

Warren's thiobarbituric acid method (1959) as modified for milk proteins by Marier *et al*. (1963) was adopted for the sialic acid determination. The solutions used in the analysis were as follows: 0.1 N H_2SO_4, 0.2 M sodium periodate (meta) in 9M phosphoric acid, a solution of 0.6 M sodium sulfate in 0.1 N H_2SO_4 to which was added sodium arsenite at a rate of 10% in terms of final concentration and a 0.5 M sodium sulfate solution to which recrystallized 2-thiobarbituric acid was added at the rate of 0.6%.

Samples of dried protein ranging in weight from 1.0 to 1.6 mg were placed in test tubes followed by the addition of 0.5 ml of 0.1 H_2SO_4. Sialic acid was released by digestion at 80°C for 40 min. To the hydrolyzed samples, 0.1 ml of the periodate solution was added. After mixing, the tubes were incubated at 25° C for 20 min. Following the addition of 1 ml of the arsenite solution, the tubes were shaken until the solution became colorless. Three milliliters of thiobarbituric acid solution were added and the tubes were shaken, tightly covered and placed in boiling water for 15 min. Upon cooling, 7 ml of cyclohexanone were added and the tubes shaken vigorously. Finally, the tubes were centrifuged in a clinical centrifuge for 10 min. The clear cyclohexanone phase was removed and read at 549 mμ in a spectrophotometer.

A standard curve was constructed using N-acetyl neuraminic acid in the range of 0.0 to 50 μg in 0.5 ml of water.

Tryptophan

This amino acid is labile to acid hydrolysis and must be determined independently from the rest.

In this study tryptophan was chemically analyzed by procedure W as described by Spies (1967). A 2 to 4 mg sample of protein was weighed directly into a small glass vial. To each sample was added 100 μl of Pronase solution which contained 10 mg of Pronase per ml of 0.1 M phosphate buffer, pH 7.5. Solutions were prepared on the day they were used. The vials were stoppered and incubated for 24 hr at 40° C.

The cooled vials were set in 25 ml Erlenmeyer flasks containing 9.0 ml of 21.2 N sulfuric acid and 30 mg of p-dimethylaminobenzaldehyde. To each vial was added 0.9 ml of 0.1 M phosphate buffer solution. The vials were tipped over and quickly mixed by swirling. The flasks were placed in the dark at 25° C for 6 hr. Then, 0.1 ml of 0.045% sodium nitrite was added. After 30 min, transmittances (%) were read at 590 mμ.

Duplicate samples of the Pronase solution, without protein, were treated and analyzed as described above. The tryptophan content of the Pronase solution was subtracted from the total tryptophan value. The blank solution contained everything but protein and Pronase.

A standard curve was constructed using 0.0 to 120 μg of tryptophan in phosphate buffer solution as described in procedures E and F by Spies and Chambers (1948).

Amino Acids

The amino acid analyses were performed on 20 and 70 hr acid hydrolysates employing a Beckman Amino Acid Analyzer, Model 120C (Moore, et al., 1958). Approximately 4 mg of dried protein were weighed into 10 ml ampoules. Six milliliters of 6 N HCl were added to the ampoules. The contents of the ampoules were frozen in dry ice-ethanol, evacuated by vacuum, allowed to melt slowly to remove gases, refrozen and sealed with an air-propane flame. The sealed ampoules were placed in an oil bath

which was heated by a recirculating oven regulated at 110° C. The sets of duplicate samples were hydrolyzed for either 20 or 70 hr.

The hydrolysates were evaporated to dryness in a 25 ml pear-shaped flask fitted to a rotary evaporator. Each residue was taken up in small amounts of deionized water and redried until all the HCl had been removed. Finally, 5 ml of a citrate-HCl buffer (pH 2.2) was added to the dried hydrolysate. An aliquot of 0.2 ml was removed from each sample for amino acid anlaysis.

Total Lipids

The lipids were extracted from the lipoprotein particles by the procedure of Folch, et al. (1957).

One gram samples were mixed with 100 ml of chloroform-methanol (2:1 v/v). After 20 min of stirring, 100 ml of a 2% KCl solution were added and stirred for 10 min. This mixture was centrifuged for 20 min at 2,000 X g. The lower lipid-containing layer was carefully removed by syringe. Anhydrous sodium sulfate was added to remove the residual moisture from the lipid extracts. Following filtration through Whatman No. 42 paper, 50 ml aliquots were evaporated in aluminum containers to determine the lipid content. Controls were analyzed to determine residual content of the solvent systems.

Total Cholesterol

This sterol was determined by modifying a method described by Bowman and Wolf (1962).

Aliquots of lipid extracts (1 to 3 mg) in chloroform-methanol were evaporated to dryness in a hot water bath. The lipids in each test tube were dissolved in 2 ml of absolute ethanol. The color was developed by adding 2 ml of a color reagent containing 8 ml of an iron stock solution diluted to 100 ml with concentrated sulfuric acid. The iron stock solution was prepared by dissolving 2.5 g $FeCl_3 \cdot 6H_2O$ in 100 ml of 87% H_3PO_4. After vigorous stirring with a Mini-Shaker, the tubes were cooled with the transmittance (%) read at 550 mμ after 20 min.

The standard curve was constructed using cholesterol (99% minimum purity) in the range of 0.0 to 55.0 μg per 2 ml.

Physical Methods

Starch-Urea Gel Electrophoresis

The procedure and equipment used in preparing and performing this type of zonal electrophoresis were adapted from the method described by Wake and Baldwin (1961).

The gel was prepared as follows: to 70 ml of stock tris-citrate buffer (92.0 g of tris-hydroxymethyl amino-methane and 12.1 g of citric acid per liter), 170 ml of deionized water and 45 g hydrolyzed starch were added with

constant stirring and heated over an open flame to 65° C. Next, 147 g of urea were slowly added with continued stirring and heating until 90° C was reached. The hot solution was poured into a two-liter filter flask and degassed by applying vacuum. The deaerated solution was poured into the gel bed and a slot former placed near the cathodic end of the cell. After aging for approximately 24 hr, the gel was prepared for the analysis.

The protein solution was prepared by adding 20 mg of protein to five-tenths milliliter of a buffer consisting of 70 ml of tris-citrate stock buffer, 170 ml water and 147 g urea. Approximately 150 µl of each sample were introduced into each gel slot. The slots were covered with a layer of mineral oil.

The buffer tanks, connected to the gel through filter paper wicks (Eaton and Dikeman No. 652), were filled with boric acid-sodium hydroxide buffer. This stock buffer contained 881 g boric acid and 190 g sodium hydroxide in 19 liters of water, adjusted to pH 8.6. The stock buffer was diluted 1:1.5 with deionized water before use. Approximately 1,200 ml of the diluted buffer were added to each buffer tank. Platinum electrodes were inserted in each buffer tank with 200-250 volts (d.c.) applied across the system for 16 hr, or the time necessary for the amino black dye marker to move 14 cm past the starting slots. This system represents discontinuous gel electrophoresis.

The gel was sliced transversely with a thin wire after the electrophoretic run was completed. The middle slice was stained for 10 min with an Amido Black staining solution containing 250 ml of water, 250 ml mechanol, 50 ml glacial acetic acid and 2 g amido black. After removing the excess stain with water, the unbound stain was removed from the gel in an electrophoretic destaining cell containing a 7% acetic acid solution.

Electron Microscopy

The samples were fixed in 1% glutaraldehyde (0.2 ml of sample containing approximately 1% membrane to 2 ml of fixative) for 15 min, diluted with distilled water, dispersed on glass slides by dipping. The samples on the glass slides were post-fixed in OsO_4 vapors for 15 min and air dried. Finally, the material was shadowed with platinum at a 3:1 angle (arc tan 1/3). The carbon film with the sample material was floated onto a water surface and picked up on copper grids. Electron microscopy was performed with a RCA EMU 3 G scope operating at 100 kV and using a 25 μ objective aperture.

Preparative Procedures for the Fat Globule Membrane Fractions

The milk used in this study was obtained from the Michigan State University dairy herd which consisted of Brown Swiss, Jersey and Holstein cows. The milk was collected at the evening milking and separated while warm.

Preparation of membranes for washing and erosion studies.--After the cream was separated, it was diluted with warm (40° C) deionized water (1:3 v/v) and reseparated. This process was repeated five times with aliquots of the cream and corresponding washings collected after each separation for subsequent analyses. The washed cream samples were standardized to a volume of 600 ml of 40% fat, cooled overnight to 5° C and churned in 2,000 ml Pyrex Erlenmeyer flasks with a rotary shaker operated at room temperature. Under these conditions the fat emulsion broke in approximately 45 min. Agitation was continued until the fat granules were gathered into ball-shaped masses about one inch in diameter. The membrane material in the buttermilk was designated as aqueous membrane whereas the material trapped in the butter granules was designated as serum membrane. The resulting aqueous membrane suspensions were filtered through a triple layer of cheese cloth to trap the small granules of butter and finally centrifuged for 0.2 hr at 2,000 X g to remove the unchurned fat globules. Each membrane sample was lyophilized and dried in a vacuum of 25 inches of Hg and over P_2O_5 at room temperature for 24 hr prior to weighing.

Total lipids were extracted from weighed aliquots of the dried membrane preparations. For this purpose four extractions with ethanol/diethyl ether mixtures of the following compositions were employed at room temperature:

40/60, 20/80, 10/90, and 0/100% (v/v). The residual proteins were dried as previously described prior to weighing. Protein percentages were estimated from weight differences.

Samples were taken from skimmilk and each cream washing and dialyzed for 24 hr with constant stirring to remove the milk salts. After these samples were dialyzed and lyophilized, 4% suspensions of the residues were analyzed for plasma proteins by starch-urea gel electrophoresis according to the method described by Wake and Baldwin (1961).

Preparation of membranes for physical and chemical studies.--The fragmented membranes were prepared from washed cream in the same manner as described in the preceding paragraphs except for the following modifications. The cream was washed 3 times, and the final fat content was adjusted to 50% before churning for the purpose of concentrating the membrane suspension. Three pellets were removed from the aqueous membrane suspension by centrifugation. These pellets were resuspended, lyophilized and dried prior to chemical analyses. For the various lipid determinations the Folch et al. (1957) extraction procedure was used to remove the total lipids from the pellets. In preparing the proteins for analysis, however, the pellet lipids were extracted with chloroform-methanol (2:1 v/v) at the rate of 100 ml of solvent per g

of membrane. These mixtures were stirred for 20 min and centrifuged for 30 min at 2,000 X g to collect the insoluble proteins. This procedure was repeated three times, and finally the protein pellets were washed with acetone before drying.

For electron microscopic examination, the wet unextracted pellets were resuspended in deionized water at 1% concentration and preserved with streptomycin and penicillin "G". The antibiotics were added at the rate of 50 mg/liter and 5,000 units/liter, respectively.

Preparation of the 7,500S, 230S and 35S pellets by centrifugation.--A scheme for fractionating the aqueous membrane suspension is presented in Figure 1 with the pellet values expressed in Svedberg units. The minimum sedimentation coefficients of the pellets were chosen so that the amount of each pellet would be sufficient for analysis and at the same time provide adequate differential fractionation. The 7,500S and 230S pellets were redispersed in 200 to 1 (v/w) deionized water before final centrifugation. This step allowed particles trapped during the initial sedimentation with coefficients less than 7,500S and 230S to be removed from the final pellets. The 35S pellet was not washed since approximately two-thirds of the membranes had already been removed in the other two pellets. Additionally, the final supernatant contained only 3 to 4% of the total membrane material.

Sedimentation rates of the pellets were determined in accordance with the equation set forth by Trautman (1961):

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

where s = the sedimentation coefficient is expressed in Svedberg units (S),

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

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

T = separation time in seconds, and

W = the angular velocity in rad/sec = $\frac{2\pi \text{ (rpm)}}{60}$.

For example, the calculations of the 7,500S pellet employing the Sorvall RC2-B with a type SS-34 rotor with the following parameters

r_a = 10.8 cm,

r_b = 5.7 cm,

rpm = 10,000, and

T = 756 sec

would yield

$$s = \frac{10^{13} \ln (10.8/5.7)}{\frac{2\pi (10,000)^2}{60} \cdot 756} = 7,500S \text{ pellet.}$$

The 230S pellet was obtained using the same equipment as above but with an increase in (T) and (rpm) as shown in Figure 1. The Beckman Model L-2 ultracentrifuge with the type 30 fixed-angle rotor was used to collect the 35 S pellet. The r_a and r_b values for this rotor are 10.5 and 5 cm, respectively.

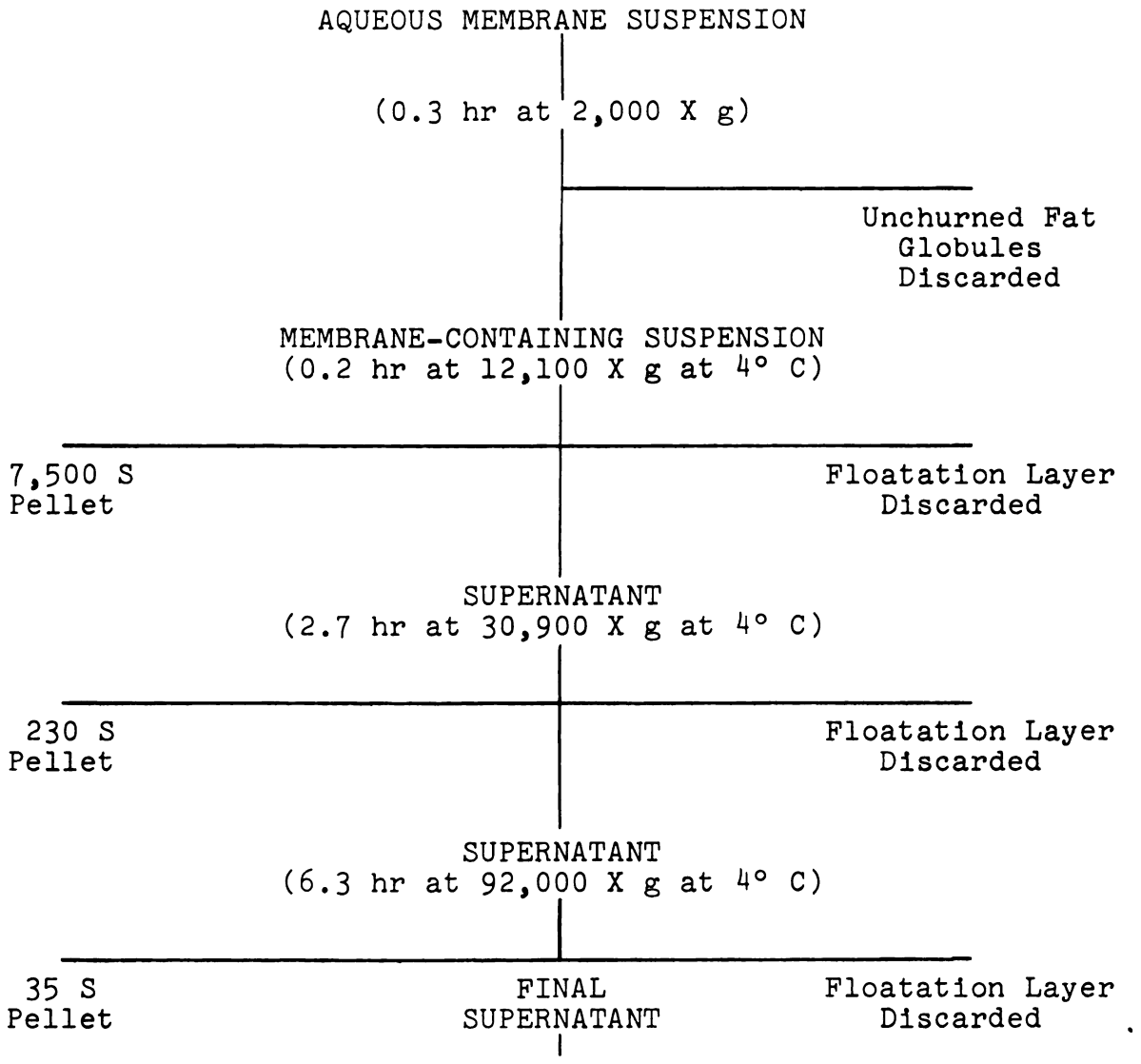


FIGURE 1.--Diagram for the isolation of the membrane pellets from eroded fat globules of thrice-washed cream. The 7,500S and 230S pellets were washed with deionized water (200:1 v/w) before final centrifugation.

RESULTS AND DISCUSSION

Preparative Procedures

The Effects of Washing the Fat Globules

The removal of the milk plasma from the fat globules before isolating the membranous material is of paramount importance. As mentioned previously in the procedural section, this separation is accomplished best by taking advantage of the density difference between the plasma and fat globules. With successive dilutions of cream with water (1:3 v/v) and reseparating, the plasma is adequately removed. This removal was monitored by taking dried samples of each washing and applying them to starch-urea gel as shown in Figure 2. Although equal amounts of a 4% concentration of the dried material from the first through the fifth washing were applied to the gel slots, the band intensities decreased progressively toward the fifth washing. Presumably, membrane lipoprotein complexes make up the majority of the material in the latter slots which do not stain well with Amino Black or migrate in starch-urea gel. Undoubtedly, these particulates are erosion products from the membrane proper.

The wash medium used in these studies was warm (40° C) deionized water. By employing warm water, the separations were essentially performed at the temperature of freshly secreted milk; thus, minimizing induced lipolysis (Thompson, et al., 1961). Actually, a saline-sucrose wash solution preserves the membrane integrity better than deionized water (Swope and Brunner, In Review). However, the need for prolonged dialysis and the threat of bacterial growth in these suspensions precluded its use.

In Figure 3 the results of washing the fat globules before isolating the membranes are presented. The concentration of the aqueous membrane material isolated from samples of cream washed from two to five times decreased from 1.05 g to 0.89 g per 100 g of fat. A loss of approximately 4% of the membrane occurred per washing. Also, the membrane protein decreased substantially from the second to third washing and then stabilized over the next several washings. At least part of this protein found in the twice-washed sample is attributable to plasma proteins since the gel electrophoretograms showed that it took three washings to remove them satisfactorily. The percentage of protein in the membrane is considerably less in Figure 3 than was found in the various pellets. However, in these washing experiments the total aqueous membrane suspension was analyzed which included high lipid fractions such as the floatation layers; consequently, the indicated amount of protein was lower.

The data show that the membrane is labile to erosion when employing dilution techniques to remove plasma material. However, washing fat globules three times with deionized warm water (1:3 dilution) will adequately remove the plasma proteins and keep the erosion at acceptable levels.

Pellet Separation by Ultracentrifugation

The fractionation scheme shown in Figure 1 separates membrane particles of different sizes and densities into various fractions by centrifugal force. By no means does this suggest that each pellet is distinctly different from the other. In fact, only an enrichment of the various particulates occurs from one pellet to the next with differential centrifugation. The pellets collected, however, were sufficiently different to warrant chemical and physical analyses, and to a reasonable degree, an interpretation of the native structure.

Other workers have employed centrifugal separation techniques to study the globule membrane in conjunction with solubilizing agents (Alexander and Lusena, 1961; Hayashi and Smith, 1965). These chemicals were avoided in this study to decrease the possibility of introducing artifacts.

Approximately 10-15% of the starting membrane material was lost in the washing of pellets, the final supernatant

and the floatation layers. The washings from the 7,500S and 230S pellets were assumed to be representative of the pellets in general and were discarded. The material in the final supernatant (approximately 4%) could not be sedimented in sufficient quantities to analyze. Perhaps of greater interest is the nature of the floatation layers. This material has physical and floatation characteristics similar to plasma chylomicrons (Wathen and Levy, 1966). Accordingly, Zittle, et al. (1956) reported that a similar substance in milk contained an exceedingly high lipid content (95%) which indicates a very low density lipoprotein. It must be assumed that these lipid-rich particles are orientated to the fat globule in an unspecified manner and desorbed during the churning process.

Chemical Analyses

The Protein-Lipid Relationship of the Various Fractions

The compositional data of the three pellets are reported in Table 1. The yield for the 7,500S pellet is considerably lower than the other two fractions. Using low centrifugal force only, the larger membrane fragments and intact membrane ghosts are sedimented. As this force is increased to 230S and finally 35S speeds, the smaller and less dense particles are removed from suspension. This latter material accounts for 80% of the total pellets.

Perhaps of more interest is the lipid distribution between pellets. The lipids increase from 17.5% in the 7,500S pellet to 55.3% in the 35S pellet. Such a variation indicates that the pellet separation is not based entirely on particle size but also on particle density.

The ratios of protein, cholesterol and phospholipids to the total lipids of each pellet are reported in Table 2. The membrane pellets are high in phospholipids which account for approximately 50% of the total lipids. Phospholipids as well as cholesterol maintain their highest level in the 7,500S pellet which contains only 17.5% lipids. As the lipids of the pellets increase--29.5% for the 230S and 55.3% for the 35S--the ratios for phospholipids and cholesterol per total lipid actually decline.

Therefore, the data lend support to the postulate that the lipids are arranged in globular micelles with the bimodal molecules of phospholipids and perhaps cholesterol located at the surface and neutral lipids toward the interior of the structure. Variations in micellular size would adequately explain the changes in the phospholipid-cholesterol/total lipids relationship of the pellets, since the surface area to volume ratios would also change.

The Protein Composition of the Different Fractions

Various chemical constituents have been identified in the lipid-extracted pellets and are reported in Table 3. Most milk proteins contain a nitrogen content between 15.0 and 16.0%, slightly higher than the membrane fractions. This difference is related to the carbohydrate moiety as revealed in the hexose, hexosamine and sialic acid content of the membrane proteins. Carbohydrates in general and sialic acid specifically appear to be a common constituent of membranes. It might be speculated that their presence in this membrane system is manifested in the aggregation or clustering of fat globules during creaming (Dunkley and Sommer, 1944).

Again, the enrichment of pellets with specific materials by differential centrifugation is amply demonstrated since the carbohydrate values range from 6.6% in the 7,500S to 8.5 and 10.1% in the 230S and 35S extracted pellets, respectively. Interestingly, the higher carbohydrate content is found in the higher lipid-containing pellets, indicating a possible relationship between these carbohydrates and the lipid micelles.

Small amounts of phosphorus are present in the extracted pellets, but whether this element is attributable to residual phospholipids, a phosphorylated protein or traces of nucleic acids is not known at present.

The amino acid residues of the pellet proteins are tabulated in Table 4. These proteins, collectively, are similar in amino acid content to other milk proteins except for a lower glutamic acid and proline and a higher arginine value. The predominant characteristic feature of these fractions is their insolubility in the usual aqueous systems employed to carry proteins. On close examination, however, the concentration of hydrophobic amino acid residues is no greater than other proteins which exhibit a high degree of solubility. It is suspected, therefore, that the sequential arrangement of amino acids is a significant factor in determining the solubility properties.

There are no striking differences in the amino acid composition of the various pellet proteins. When all three fractions are compared on mole basis (Table 5), the 35S amino acid content exhibit more variation than the other two pellets. Seemingly, this change suggests a greater selectivity in protein composition for this high lipid-containing fraction. A meaningful interpretation of these results is not possible, however, since the pellets at present are undefined protein mixtures. Also, it must be remembered that proteins do not necessarily show a sizable variation in their amino acid content in order to reflect considerable differences in their physical and chemical properties. Consequently, the 7,500S and 230S

pellets may be actually more unlike than their amino acid composition would seem to indicate.

Proposed Structure for the Fat Globule Membrane

Supporting Evidence for a Protein-Lipoprotein Structure

In view of the data already presented, the fat globule membrane appears to consist of a proteinaceous layer(s) which serves internally as a covering for the glyceride droplet and externally as adsorption sites for lipoprotein micelles. The characteristic "insolubility" of this major membrane protein gives credence to such a structural role. Like other structural proteins, its intermolecular binding forces have not allowed for definitive physical characterization (Harwalkar and Brunner, 1965).

King (1955) postulated that these proteins were held to the lipid surface of the globule by electrostatic bonding by the polar ends of phospholipids. Recently, Patton and Fowkes (1967) have proposed that the protein envelopment of the droplet can be adequately explained on the basis of London-van de Waals forces which are estimated to achieve an attraction force of one atmosphere when the separating distance between the membrane and fat droplet is reduced to 20 Å. Therefore, electrostatic bonding would be of little consequence under these

conditions. Morton (1954) gave additional evidence for a protein layer(s) when he proposed that a continuous protein membrane surrounds the fat globule to which were adsorbed microsomal lipoprotein particles. By employing sodium deoxycholate as a solubilizing agent on the intact globules, Hayashi and Smith (1965) discovered a water-insoluble matrix to which water-soluble lipoproteins were attached.

Substantial evidence is now available to verify the presence of loosely adsorbed lipoprotein particles on the membrane proper. The washing experiments, discussed earlier in this investigation, show a steady erosion of the membrane during the isolation procedures. Koops et al. (1958), examining the membrane thickness with electron microscopy, found that it was dependent on prior treatment of the cream. The membrane was noticeably thicker in the case of cream allowed to rise naturally. Any form of agitation apparently causes a migration of membrane phospholipids (Greenbank and Pallansch, 1961). Even the effect of heating results in approximately 20% lipid phosphorus dispersion to the aqueous phase (Koops and Tarassuk, 1959). Recently, Chein and Richardson (1967a) confirmed the presence of a substantial neutral lipid content in these migrating lipoprotein particles.

The actual structure required for lipoprotein stability is a matter of conjecture at this point.

However, experimenting with various mixtures of lecithin, cholesterol and saponin, Lucy and Glauert (1964) observed a variety of molecular arrangements as interpreted with the aid of electron microscopy. They concluded that only the formation of small globular micelles would satisfactorily explain these organized units. Eley and Hedge (1956) had previously substantiated that lipid micelles bind proteins primarily through hydrogen and electrostatic bonding. The same forces should also be applicable to the lipoproteins of the fat globule membrane.

The chemical composition and physical properties of the pellet material strongly imply the presence of adsorbed lipoprotein of various sizes which are similar in many respects to those found in chyle and blood serum. Cook and Martin (1962) have surveyed over 40 lipoprotein fractions and have discovered a definite relationship between the neutral lipid, phospholipids and protein of these particles. For instance, when the neutral lipid increases in these lipoproteins, the phospholipid decreases. As the protein content rises above 30%, the neutral lipid phospholipids are present in similar proportion. All this information seems to indicate the formation of globular micelles with a surface area relatively rich in phospholipids and proteins. In the case of the membrane pellets, the same type compositional relationship was found likely to exist.

Interpretation of Electron Micrographs of 7,500S, 230S and 35S Pellets

To propose a membrane structure based entirely on electron microscopy would be misleading and perhaps erroneous. Korn (1966) has aptly pointed out the limitations of this analytical procedure and suggests that chemical differences among membranes are more definitive and more significant than the similar appearance of membranes in electron micrographs. For this reason, the author has chosen to use three areas of support for elucidating the fat globule membrane structure: (1) the chemical data already presented in this investigation; (2) research contributions of other workers as reviewed in the literature section; (3) examination of the 7,500S, 230S and 35S membrane pellets by electron microscopy. Hopefully, a reasonable membrane model can be drawn by integrating these areas. Therefore, the schematic interpretation of each pellet is presented alongside an electron micrograph of the corresponding pellet primarily as an illustrative aid. Regions that best represent or describe these interpretations are indicated by markers, but they do not necessarily reflect in absolute terms the pictorial interpretations.

A membrane ghost from the 7,500S pellet is shown in Figure 4. This pellet is characterized by large, low lipid-containing fragments. A rough overlapping membrane section is clearly visible in the electron micrograph.

This structure is interpreted in the schematic representation as consisting of a basal layer of proteins with a few small and intermediate sized, adsorbed lipoprotein micelles. The micelles would easily account for the high phospholipid-cholesterol/total lipid ratio (see Table 2).

In Figure 5 an eroded membrane and a few irregular shaped particles are present in the electron micrograph, presumably originating from the membrane proper. Rough and smooth sections are quite noticeable which may represent areas of adsorbed and desorbed lipoproteins. One of these areas might reflect the opposite side of the membrane. In either case, the proposed protein-lipoprotein complex is still tenable. The membrane fragments in the 230S pellet are generally smaller and less dense than those found in the 7,500S pellet. The total lipid content rises with an accompanying change in the phospholipid-cholesterol/total lipid ratio.

Finally in the high-speed 35S pellet (Figure 6), small membrane fragments exhibiting definite structural features are found individually and in clusters. The total lipid, phospholipid and cholesterol content suggests that they are primarily large micelles. The "insoluble" membrane protein is still present in this fraction denoting the inclusion of basal material. The carbohydrate content has the highest value of any fraction,

indicating that at least one of the proteins associated with the micelles has a high carbohydrate moiety (see Appendix).

In the foregoing discussion various aspects of membrane and lipoprotein structure have been reviewed with particular emphasis placed on the forces required for their formation and stability. The physical behavior and quantitative analyses of the fat globule membrane imply that these same forces are also responsible for its integrity. The schematic interpretations (Figures 4-6), however, represent the simplest possible arrangement for this material. Visually, this membrane is portrayed as a proteinaceous layer held tightly to the lipid core by London-van der Waals forces. Externally, lipoprotein micelles of various size and composition are adsorbed to this layer through the influence of electrostatic and hydrogen bonding.

Even with this plausible model of the fat globule membrane, many questions concerning its structure and composition remain unanswered. For example, the basal protein might be arranged in a mosaic pattern leaving exposed interstices by which the lipoproteins are anchored to the lipid core. The data presented could accommodate such a model with slight modification in chemical bonding. Additionally, the lipoprotein micelles

are exceedingly rich in the enzymes xanthine oxidase and alkaline phosphatase for which there is no adequate explanation. Finally, no distinction has been made between the lipoproteins of the various fractions of milk. All the lipoproteins may or may not be derived from the membrane complex.

In conclusion, the fat globule membrane must be considered a heterogeneous system which contains a structural protein, lipoprotein micelles and other substances closely identified with the secretory cells of the mammary tissue. The precise nature of this material has not yet been defined in specific terms, and additional research will be required to completely unravel the mystery surrounding it.

TABLE 1.--Appearance and composition of the various membrane pellets obtained from fifteen hundred milliliters of fat globules.[a]

Pellet	Mass of pellet (g)	Mass of lipid (g)	Lipids in pellet (%)	Appearance
7,500 S	1.77	0.31	17.51	white
230 S	4.44	1.31	29.50	white-brown
35 S	3.13	1.73	55.27	brown
TOTAL	9.34	3.35	35.87	

[a]Three liters of 50% washed cream.

TABLE 2.--Relationship of protein, cholesterol and phospholipid content to the total lipids for each fraction.[a]

Pellet	Protein / lipid	Cholesterol / lipid	Phospholipid[b] / lipid
7,500 S	4.71	0.041	0.632
230 S	2.39	0.030	0.532
35 S	0.81	0.028	0.421

[a]Expressed as component ratios.

[b]Phospholipid P X 25.

TABLE 3.--Composition of the lipid-extracted pellets.[a]

Constituent	7,500 S	230 S (%)	35 S
Nitrogen	14.14	14.05	14.01
Phosphorus	0.16	0.17	0.23
Hexose	2.82	3.49	4.15
Hexosamine	2.48	3.22	4.17
Sialic acid	1.29	1.74	1.77

[a]Protein residues.

TABLE 4.--Amino acid composition of the protein fractions.[a]

Residue	7,500 S	230 S	35 S
	(g of residue/100 g protein)[b,c]		
Lysine	6.6	6.6	5.8
Histidine	2.1	2.3	2.7
Arginine	6.6	6.5	5.5
Aspartic acid	7.7	7.6	9.0
Threonine	4.7	4.9	5.8
Serine	5.4	5.6	5.2
Glutamic acid	11.3	11.0	10.6
Proline	5.1	5.4	4.3
Glycine	3.2	3.3	3.5
Alanine	3.9	3.9	4.0
1/2 Cystine	1.5	1.4	1.9
Valine	4.5	4.4	4.5
Methionine	1.9	1.4	1.2
Isoleucine	4.4	4.3	4.5
Leucine	8.2	8.1	7.4
Tyrosine	3.6	3.5	3.9
Phenylalanine	5.3	5.5	5.3
Tryptophan	2.2	2.3	2.4
Amino acid residue (weight %)	88.2	88.0	87.5
Total carbohydrate (weight %)	6.6	8.5	10.1
P as H_2PO_3	0.4	0.4	0.6
Total (weight %)	95.2	96.9	98.2

[a]Pellet protein residues following lipid extraction.

[b]Weight percentage of each amino acid residue based on a nitrogen content of 14.14%, 14.05%, and 14.01% for each respective pellet.

[c]Amino acid content based on duplicate analyses of 20 and 70 hr digestion with values corrected for destruction during hydrolysis according to the equation given by Hirs, Stein, and Moore (1954).

TABLE 5.--Amino acid composition of the protein fractions expressed in moles.[a]

Residue	7,500 S	230 S	35 S
	(moles/1000 moles)		
Lysine	65.2 ± 0.8	65.0 ± 0.2	57.5 ± 0.1
Histidine	19.6 ± 0.8	21.1 ± 0.4	25.2 ± 0.1
Arginine	53.7 ± 0.5	52.4 ± 0.2	44.3 ± 0.4
Aspartic acid	84.1 ± 0.1	84.1 ± 0.6	98.4 ± 1.6
Threonine	58.3 ± 0.3	61.4 ± 0.1	72.6 ± 0.3
Serine	77.8 ± 0.5	81.5 ± 1.2	75.3 ± 0.9
Glutamic acid	110.5 ± 0.3	107.6 ± 0.2	103.6 ± 0.5
Proline	65.7 ± 0.7	70.3 ± 1.3	56.6 ± 0.7
Glycine	71.6 ± 0.6	72.5 ± 0.4	78.5 ± 0.4
Alanine	69.9 ± 0.8	69.4 ± 0.4	71.0 ± 0.1
1/2 Cystine	18.8 ± 0.9	17.1 ± 0.3	23.7 ± 1.2
Valine	57.6 ± 0.6	55.7 ± 0.8	57.1 ± 0.3
Methionine	18.4 ± 0.2	13.9 ± 0.6	12.1 ± 0.2
Isoleucine	49.3 ± 0.5	47.9 ± 0.5	50.4 ± 0.7
Leucine	91.2 ± 0.3	90.1 ± 0.5	82.7 ± 1.4
Tyrosine	28.1 ± 0.5	27.2 ± 0.2	30.1 ± 0.4
Phenylalanine	45.2 ± 0.3	47.4 ± 0.2	45.1 ± 0.6
Tryptophan	15.0 ± 0.1	15.4 ± 0.1	15.8 ± 0.2
	$\Sigma N = 1000$		

[a]Amino acid content based on duplicate analyses of 20 and 70 hr digestion with values corrected for destruction during hydrolysis according to the equation given by Hirs, Stein, and Moore (1954). The deviation from the mean is shown for each value.

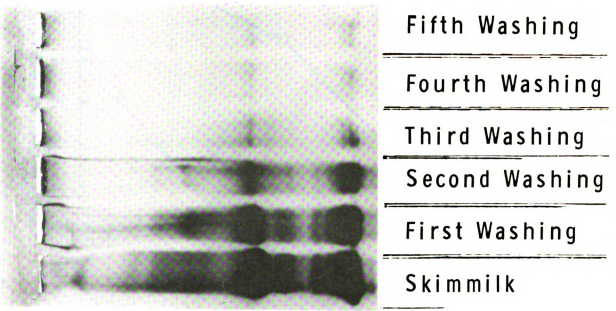


Figure 2.--Starch-urea gel electrophoretograms of skimmilk and cream washings. Samples were applied as 4% concentrations.

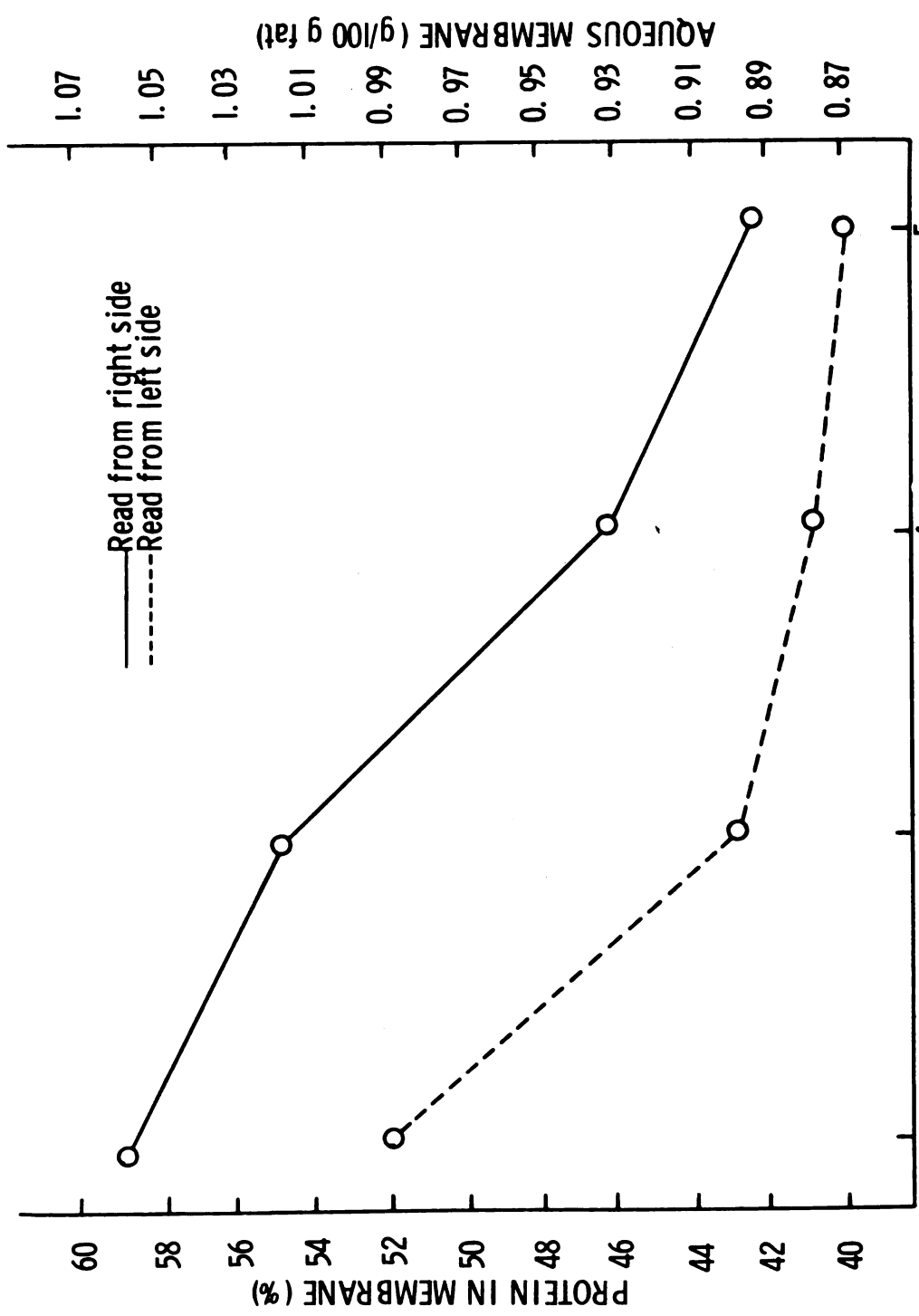
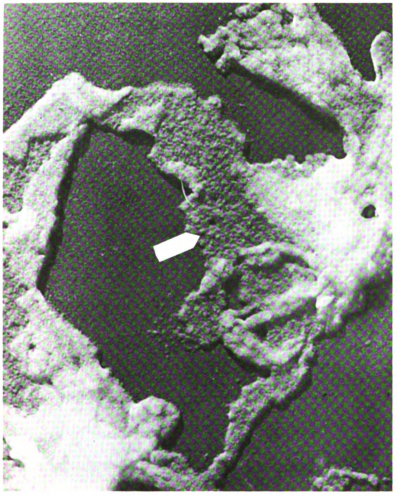


Figure 3.--Yield and gross composition of the aqueous membrane fractions recovered from aliquots of a two to five times washed cream.



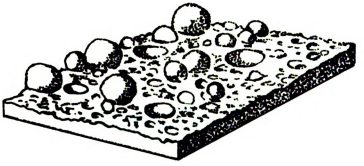


Figure 4.--Electron micrograph and a corresponding schematic interpretation of a torn fat globule membrane ghost isolated from the 7,500S pellet. The marker indicates an area of membrane containing adsorbed lipoprotein micelles. 58,000X.

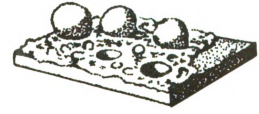
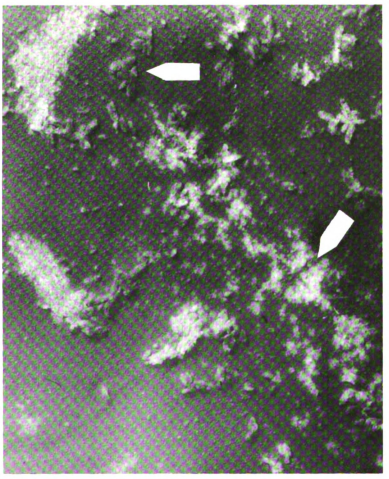


Figure 5.--Electron micrograph and a corresponding schematic interpretation of a fragmented fat globule membrane isolated from the 230S pellet. The marker points to a rough and smooth area of the membrane. 58,000X.



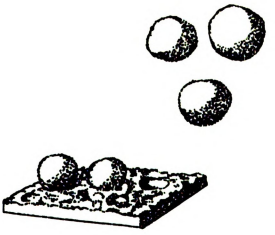


Figure 6.--Electron micrograph and a corresponding schematic interpretation of small membrane fragments isolated from the 35S pellet. The markers, individually, point to large desorbed lipoprotein micelles and to less defined areas probably containing the basal membrane layer. 58,000X.

SUMMARY

1. Washing and reseparating fat globules three times with three volumes of deionized water per volume of cream will adequately remove the milk plasma.
2. Material was lost from the membrane proper at an uniform rate during the preparative procedures.
3. The total lipids increased from the 7,500S pellet (17.5%) and reached their highest value in the 35S (55.3%) pellet.
4. The total lipids of the 7,500S pellet contained 4% cholesterol and 63% phospholipids, but these values decreased to 2.8% cholesterol and 42.0% phospholipids in the 35S pellet.
5. The proteins from the 7,500S pellet contained 6.6% carbohydrates while the 230S and 35S contained 8.5 and 10.1%, respectively.
6. The pellet proteins contained more arginine and less glutamic acid and proline than other milk proteins.
7. The protein pellet from the 35S exhibited better solubilizing properties than the 7,500S and 230S lipid-extracted pellets.
8. A membrane model is proposed utilizing the physical and chemical data collected during this study.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Abderhalden, E., and Völtz, W.
   1909. Beitrag zur Kenntnis der Zusammensetzung und der Natur Hüllen der Milchkügelchen. Hoppe-Seyl. Z., 59:13.

2. Alexander, K. M., and Lusena, C. V.
   1961. Fractionation of the lipoproteins of the fat globule membrane from cream. J. Dairy Sci., 44:1414.

3. Andrews, P., Bray, R. C., Edwards, P., and Shooter, K.V.
   1964. The chemistry of xanthine oxidase. II. Ultracentrifuge and gel-filteration studies on the milk enzyme. Biochem. J., 93:627.

4. Ascherson, F. M.
   1840. On the physiological utility of the fats and a new theory of cell formation based on their cooperation and suggested by several new facts. A translation by Emil Hatschek in The Foundations of Colloid Chemistry, E. Benn, Ltd., London, 1925.

5. Avis, P. G., Bergel, F., Bray, R. C., James, W. F., and Shooter, K. V.
   1956. Cellular constituents. The chemistry of xanthine oxidase. Part II. The homogeneity of crystalline metalloflavoprotein fractions. J. Chem. Soc., 1212.

6. Babcock, S. M.
   1885. A study of the fat globules of milk. N. Y. (Geneva) Agr. Expt. Sta., 4th. Ann. Rpt. 293.

7. Babcock, S. M.
   1889. Fibrin in milk.
   Wis. Agr. Expt. Sta. 6th. Ann. Rpt., 63.

8. Baillie, M. J., and Morton, R. K.
   1958a. Comparative properties of microsomes from cow's milk and from mammary gland. I. Enzymic activities. Biochem. J., 69:35.

9. Baillie, M. J., and Morton, R. K.
   1958b. Comparative properties of microsomes from cow's milk and from mammary gland. 2. Chemical composition. Biochem. J., 69:44.

10. Bargmann, W., Fleischhauer, K., and Knoop, A.
1961. Über die Morphologie der Milchsekretion. II. Zugleich eine Kritik am Schema der Sekretionsmorphologie. Z. Zellforsch. 53:545.

11. Bargmann, W., and Knoop, A.
1959. Über die Mrophologie der Michsekretion. I. Licht- und Electronenmikroskopische Studien an der Milchdrüse der Ratte. Z. Zellforsch. 49:344.

12. Bauer, H.
1911. Untersuchungen über Oberflächenspannungsverhältnisse in der Milch und über die Natur der Hüllen der Milchfettkügelchen. Biochem. Z., 32:362.

13. Bingham, E. W., Jasewicz, L., and Zittle, C. A.
1961. Acid phosphatase in cream and skimmilk. J. Dairy Sci., 44:1247.

14. Bingham, E. W., and Zittle, C. A.
1964. Ribonuclease of bovine milk: Purification and properties. Arch. Biochem. Biophys., 106:235.

15. Bowman, R. E., and Wolf, R. C.
1962. A rapid and specific ultramicro method for total serum cholesterol. Clin. Chem., 8:302.

16. Brunner, J. R.
1962. Protein-lipid interactions and their relation to the physical-chemical stability of concentrated milk. A review. J. Dairy Sci., 45:943.

17. Brunner, J. R.
1965. Physical equilibria in milk: The lipid phase. In Fundamentals of Dairy Chemistry (B. H. Webb and A. H. Johnson, eds.). Avi Publishing Co., Inc., Westport, Conn.

18. Brunner, J. R., Duncan, C. W., and Trout, G. M.
1953a. The fat-globule membrane of nonhomogenized and homogenized milk. I. The isolation and amino acid composition of the fat-membrane proteins. Food Res., 18:454.

19. Brunner, J. R., Duncan, C. W., and Trout, G. M.
1953c. The fat-globule membrane of nonhomogenized and homogenized milk. III. Differences in the sedimentation diagrams of the fat-membrane proteins. Food Res., 18:469.

20. Brunner, J. R., and Herald, C. T.
1958. Some electrophoretic characteristics of two protein components of the fat-globule membrane of normal milk.
J. Dairy Sci., 41:1489.

21. Brunner, J. R., Lillevik, H. A., Trout, G. M., and Duncan, C. W.
1953b. The fat-globule membrane of nonhomogenized and homogenized milk. II. Differences in the electrophoretic patterns of the fat-membrane proteins. Food. Res., 18:463.

22. Cerbulis, J., and Zittle, C. A.
1965. Lipids associated with acid-precipitated casein. J. Dairy Sci., 48:1154.

23. Chien, H. C., and Richardson, T.
1967a. Gross structure of the fat globule membrane of cow's milk. J. Dairy Sci., 50:451.

24. Chien, H. C., and Richardson, T.
1967b. Gross structure of the fat globule membrane of cow's milk. II. Distribution of amino acids, hexosamines, ribonucleic acid, copper and iron. J. Dairy Sci., 50:1868.

25. Cook G. M. W., Heard, D. H., and Seaman, G. V. F.
1961. Sialic acids and the electrokinetic charge of the human erythrocyte. Nature, 191:44.

26. Cook, W. H., and Martin, W. G.
1962. Composition and properties of soluble lipoproteins in relation to structure.
Can. J. Biochem. Physiol. 40:1273.

27. Cornwell, D. G., and Horrocks, L. A.
1964. Protein-lipid complexes. In Symposium on Foods: Proteins and Their Reactions (H. W. Schultz and A. F. Anglemier, eds.), The Avi Publishing Co. Inc., Westport, Conn.

28. Coulson, E. J., and Stevens, H.
1950. The serological relationship of bovine whey albumin to serum albumin. J. Biol. Chem., 187:355.

29. Danielli, J. F., and Davson, H.
1934. A contribution to the theory of permeability of thin films. J. Cellular Comp. Physiol., 5:495.

30. Davies, W. L.
1933. The mode of combination and distribution of traces of heavy metals in dairy products. J. Dairy Res., 4:255.

31. DeRobertis, E. D. P., Nowinski, W. W., and Saez, F. A.
1965. Cell Biology. W. B. Saunders Co., Phila., Penn.

32. Dixon, F. J., Weigle, W. O., and Vazquez, J. J.
1961. Metabolism and mammary secretion of serum proteins in the cow. Lab. Invest. 10:216.

33. Dowben, R. M., Brunner, J. R., and Philpott, D. E.
1967. Studies on milk fat globule membranes. Biochim. Biophys. Acta, 135:1.

34. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F.
1956. Colorimetric method for determination of sugars and related substances. Anal. Chem., 28:350.

35. Dunkley, W. L., and Sommer, H. H.
1944. The creaming of milk. Wis. Agr. Expt. Sta. Res. Bull., 151.

36. Eley, D. D., and Hedge, D. G.
1956. Protein interactions with lecithin and cephalin monolayers. J. Colloid Sci., 11:445.

37. Emmelot, P., Bos, C. J., Benedetti, E. L., and Rümke, P.
1964. Studies on plasma membranes. I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. Biochim. Biophys. Acta, 90:126.

38. Erickson, D. E., Dunkley, W. L., and Smith, L. M.
1964. Tocopherol distribution in milk fractions and its relation to antioxidant activity. J. Food Sci., 29:269.

39. Folch, J., Lees, M., and Sloane-Stanley, G. H.
1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226:497.

40. Folley, S. J., and White, P.
1936. The effects of thyroxine on milk secretion and on the phosphatase of blood and milk of the lactating cow. Proc. Roy. Soc. (London), Ser. B, 120:346.

41. Glascock, R. F., Welch, V. A., Bishop, C., and Davies, T.
1964. The contribution of serum beta-lipoprotein triglycerides to milk fat in the dairy cow. Proc. Symp. Radioisotopes Amin. Nutr. Physiol., 253.

42. Glascock, R. F., Welch, V. A., Bishop, C., Davies, T., Wright, E. W., and Noble, R. C.
1966. An investigation of serum lipoproteins and of their contribution to milk fat in the dairy cow. Biochem. J. 98:149.

43. Graham, W. R. Jr., Jones, T. S. G., and Kay, H. D.
1936. The precursors in cow's blood of milk fat and other milk constituents. Proc. Roy. Soc. (London), Ser. B, 120:330.

44. Graham, W. R. Jr., and Kay, H. D.
1933. Phosphorus compounds of milk. V. The phosphorus partition in milk with preliminary observations on milk phosphatase. J. Dairy Res., 5:54.

45. Green, D. E., and Tzagoloff, A.
1966. Role of lipids in the structure and function of biological membranes. J. Lipid Res., 7:587.

46. Greenbank, G. R., and Pallansch, M. J.
1961. Migration of phosphatides in processing dairy products. J. Dairy Sci., 44:1597.

47. Hare, J. H., Schwartz, D. P., and Weese, S. J.
1952. The amino acid composition of the fat globule membrane protein of milk. J. Dairy Sci., 35:615.

48. Hattori, K.
1925. Membrane of the fat globule in milk. J. Pharm. Soc., Japan, No. 516:123. (Original not seen. Cited by C. T. Herald, Ph.D. thesis. Michigan State University. 1956).

49. Hayashi, S., Erickson, D. R., and Smith, L. M.
1965. Membranous material of bovine milk fat globules. II. Some physical and enzymic properties of the deoxycholate-released lipoproteins. Biochemistry, 4:2557.

50. Hayashi, S., and Smith, L. M.
1965. Membranous material of bovine milk fat globules. I. Comparison of membranous fractions released by deoxycholate and by churning. Biochemistry, 4:2550.

51. Herald, C. T., and Brunner, J. R.
1957. The fat-globule membrane of normal cow's milk. I. The isolation and characteristics of two membrane-protein fractions. J. Dairy Sci., 40:948.

52. Herald, C. T., Brunner, J. R., and Bass, S. T.
1957. A spectrographic analysis of two protein fractions of the fat-globule membrane of normal cow's milk. J. Dairy Sci., 40:446.

53. Hirs, C. H. W., Stein, W. H., and Moore, S.
1954. The amino acid composition of ribonuclease. J. Biol. Chem., 211:941.

54. Ibbott, F. A., and Abrams, A.
1964. The phospholipids in membrane ghosts from Streptococcus faecalis protoplasts. Biochemistry, 3:2008.

55. Jack, E. L., and Dahle, C. D.
1937. The electrokinetic potential of milk fat. III. Relation to the fat globule membrane. J. Dairy Sci., 20:637.

56. Jackson, R. H., Coulson, E. J., and Clark, W. R.
1962. The mucoprotein of the fat/plasma interface of cow's milk. I. Chemical and physical characterization. Arch. Biochem. Biophys., 97:373.

57. Jenness, R., and Palmer, L. S.
1945a. Substances adsorbed on the fat globules in cream and their relationship to churning. V. Composition of the "membrane" and distribution of the adsorbed substances in churning. J. Dairy Sci., 28:611.

58. Jenness, R., and Palmer, L. S.
1945b. Substances adsorbed on the fat globules in cream and their relationship to churning. VI. Relation of the high-melting triglyceride fraction to butterfat and the "membrane." J. Dairy Sci., 28:653.

59. Johansen, P. G., Marshall, R. D., and Neuberger, A.
1960. Carbohydrates in proteins. 2. The hexose, hexosamine, acetyl and amide-nitrogen content of hen's-egg albumin. Biochem. J. 77:239.

60. King, N.
1955. The Milk Fat Globule Membrane and Some Associated Phenomena. Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England.

61. King, R. L., and Dunkley, W. L.
1959. Relation of natural copper in milk to incidence of spontaneous oxidized flavor. J. Dairy Sci., 42:420.

62. King, R. L., Luick, J. R., Litman, I. I., Jennings, W. G., and Dunkley, W. L.
1959. Distribution of natural and added copper and iron in milk. J. Dairy Sci., 42:780.

63. Knoop, E., Wortman, A., and Knoop, A. M.
1958. Die Hüllen der Milchfettkügelchen im Elektronenmikroskopischen Bild. Naturwissenschaften, 45:418.

64. Kon, S. K., Mawson, E. H., and Thompson, S. Y.
1944. Relation of the concentration of vitamin A, carotenoids and cholesterol in milk fat to the size of the fat globules. Nature 154:82.

65. Kono, T., and Colowick, S. P.
1961. Isolation of skeletal muscle cell membrane and some of its properties. Arch. Biochem. Biophys., 93:520.

66. Koops, J., and Tarassuk, N. P.
1959. The effect of various processing treatments on the partition of phosphatides between the fat phase and the milk plasma. Neth. Milk Dairy J., 13:180.

67. Koppejan, C. A., and Mulder, H.
1953. The distribution of copper and iron over the various phases of milk. Intern. Dairy Congr. XIII, The Hague, 3, 1402.

68. Korn, E. D.
1966. Structure of biological membranes. Science, 153:1491.

69. Larson, B. L.
1965. Biosynthesis of the milk proteins. J. Dairy Sci., 48:133.

70. Larson, B. L., and Gillespie, D. C.
1957. Origin of the major specific proteins in milk. J. Biol. Chem., 227:565.

71. Long, C.
1961. In Biochemists' Handbook. (C. Long, ed.) D. VanNostrand Co., Inc., Princeton, N. J.

72. Lucy, J. A., and Glauert, A. M.
1964. Structure and assembly of macromolecular lipid complexes of globular micelles. J. Mol. Biol., 8:727.

73. Luick, J. R.
1961. Synthesis of milk fat in the bovine mammary gland. J. Dairy Sci., 44:652.

74. Marier, J. R., Tessier, H., and Rose, D.
1963. Sialic acid as an index of the k-casein of bovine milk. J. Dairy Sci., 46:373.

75. Matsushita, S., Ibuki, F., Mori, T., and Hata, T.
1965. Phosphodiesterase in microsomes from bovine milk. Agr. Biol. Chem., 29:436.

76. McCarthy, R. D., Ghiardi, F. L. A., and Patton, S.
1965. The conversion of stearic acid to oleic in freshly secreted milk. Biochem. Biophys. Acta, 98:216.

77. McMeekin, T. L., and Polis, B. D.
1949. Milk proteins. In Advances in Protein Chemistry. Vol. 5. (M. L. Anson, John T. Edsall, and Kenneth Bailey, eds.), Acad. Press, Inc., New York.

78. Moore, S., and Spackman, D. H., and Stein, W. H.
1958. Chromatography of amino acids on sulfonated polystyrene resins. Anal. Chem., 30:1185.

79. Morton, R. K.
1953a. Microsomal particles of normal cow's milk. Nature, 171:734.

80. Morton, R. K.
1953b. Alkaline phosphatase of milk. I. Association of the enzyme with a particulate lipoprotein complex. Biochem. J., 55:786.

81. Morton, R. K.
1953c. Alkaline phosphatase of milk. II. Purification of the enzyme. Biochem. J., 55:795.

82. Morton, R. K.
1954. The lipoprotein particles in cow's milk. Biochem. J. 57:231.

83. Mulder, H.
1957. The surface layers of milk fat globules. I. Neth. Milk Dairy J., 11:197.

84. Mulder, H., and Menger, J. W.
1958. The surface layers of milk fat globules. III. The protein content of the surface layers. Neth. Milk Dairy J., 12:1.

85. Mulder, H., Menger, J. W., and Koops, J.
1957. The surface layers of milk fat globules. II. The phospholipid content of the surface layers. Neth. Milk Dairy J., 11:263.

86. Mulder, H., and Zuidhof, T. A.
1958. The surface layers of milk fat globules. IV. The cholesterol content (free and esterified cholesterol) of the surface layers. Neth. Milk Dairy J. 12:173.

87. Muralt, G. von, Gugler, E., and Roulet, D. L. A.
1964. Immuno-electrophoretic studies of the proteins of human milk and colostrum. Immuno-Electrophoretic Analysis (P. Grabar and P. Burtin, eds.), Elsevier Publishing Co., New York.

88. Novikoff, A. B., Podber, E., Ryan, J., and Noe, E.
1953. Biochemical heterogeneity of the cytoplasmic particles isolated from rat liver homogenate. J. Histochem. Cytochem. 1:27.

89. Owen, E. C., and Hart, L. I.
1962. Xanthine oxidase in human milk. The nutrition society's meeting for original communications. Abstracts of communications, Proc. Nutr. Soc., 21:15.

90. Paape, M. J., and Tucker, H. A.
1966. Somatic cell content variation in fraction-collected milk. J. Dairy Sci., 49:265.

91. Palmer, L. S.
1944. The structure and properties of the natural fat globule "membrane." J. Dairy Sci., 27:471.

92. Palmer, L. S., and Samuelsson, E.
1924. The nature of the substances adsorbed on the surface of the fat globules in cow's milk. Proc. Soc. Exptl. Biol. Med., 21:537.

93. Palmer, L. S., and Tarassuk, N. P.
1936. The effect of the adsorption "membrane" around the fat globules on curd tension of cow's milk. J. Dairy Sci., 19:323.

94. Palmer, L. S., and Tarassuk, N. P.
1940. Physical and chemical properties of the fat globule adsorption "membrane." II. Nature and origin of surface active materials involved in curd tension reduction and prevention of rennet clot of cow's milk by "membranes" from natural and synthetic creams. J. Dairy Sci., 23:861.

95. Palmer, L. S., and Wiese, H. F.
1933. Substances adsorbed on the fat globules in cream and their relation to churning. II. The isolation and identification of adsorbed substances. J. Dairy Sci., 16:41.

96. Parpart, A. K., and Ballentine, R.
1952. Molecular anatomy of the red cell plasma membrane. In Modern Trends in Physiology and Biochemistry (E. S. G. Barron, ed.), Acad. Press., New York.

97. Patton, S., Durdan, A., and McCarthy, R. D.
1964. Structure and synthesis of milk fat. VI. Unity of the phospholipids in milk. J. Dairy Sci., 47:489.

98. Patton, S., and Fowkes, F. M.
1967. The role of the plasma membrane in the secretion of milk fat. J. Theoret. Biol., 14:274.

99. Patton, S., and Keeney, P. G.
1958. The high-melting glyceride fraction from milk fat. J. Dairy Sci., 41:1288.

100. Patton, S., McCarthy, R. D., and Dimick, P. S.
1965. Structure and synthesis of milk fat. IX. Site of lipid synthesis in freshly secreted milk. J. Dairy Sci., 48:1389.

101. Payens, T. A. J.
1959. Model experiments on lipoprotein interaction in the fat globule membrane of milk. Neth. Milk Dairy J. 13:237.

102. Payens, T. A. J., Koops, J., and Mogot, M. F. K.
1965. Adsorption of euglobulin on agglutinating milk fat globules. Biochim. Biophys. Acta, 94:576.

103. Peereboom, J. W. C.
1966. Studies on alkaline milk phosphatase. I. Non-identity of raw and reactivated alkaline phosphatases from cream. Neth. Milk Dairy J., 20:9.

104. Perlman, J. L.
1935. The distribution of phospholipids in cream. J. Dairy Sci., 18:113.

105. Polis, B. D., and Shmukler, H. W.
1950. Aldolase in milk. J. Dairy Sci., 33:619.

106. Polis, B. D., Shmukler, H. W., and Custer, J. H.
1950. Isolation of a crystalline albumin from milk. J. Biol. Chem., 187:349.

107. Rhodes, D. N., and Lea, C. H.
1958. On the composition of the phospholipids of cow's milk. J. Dairy Res., 25:60.

108. Rimpila, C. E., and Palmer, L. S.
1935. Substances adsorbed on the fat globules in cream and their relation to churning. IV. Factors influencing the composition of the adsorption "membrane." J. Dairy Sci., 18:827.

109. Rodkey, F. L., and Ball, E. G.
1946. A rapid test for distinguishing human from cow's milk based upon the difference in their xanthine oxidase content. J. Lab. Clin. Med., 31:354.

110. Roland, F.
1956. Die Quantitative Bistimmung der Hülleneiweiss-Substanz der Milchfettkügelchen. Milchwissenschaft, 11:85.

111. Rondle, C. J. M., and Morgan, W. T. J.
1955. The determination of glucosamine and galactosamine. Biochem. J. 61:586.

112. Samuelsson, E. G.
1966. The copper content in milk and the distribution of copper to some various phases of milk. Milchwissenschaft, 21:335.

113. Sasaki, R., and Koyama, S.
1956. On the fat globule membrane materials of cow's milk. I. Isolation of membrane protein and its electrophoretic properties. Intern. Dairy Congr. XIV, Rome, 2:381.

114. Sasaki, R., and Koyama, S.
1959. On the fat-globule membrane materials of cow's milk. III. Electrophoretic and enzymatic properties of membrane lipoprotein and its relation to other lipoproteins in cream. Intern. Dairy Congr. XV, London, 1:308.

115. Schwarz, G.
1947. Die Hüllen der Fettkügelchen. Neue Molkereiztg. Hildesheim, 2:33.

116. Sharp, P. F., and Hand, D. B.
1940. Amount of flavoprotein on the surface of milk fat globules. N. Y. (Cornell) Agr. Expt. Sta. Rpt., 121.

117. Smith, G. J., and Dunkley, W. L.
1961. The mechanism of spontaneous peroxidation in milk. J. Dairy Sci., 44:1152.

118. Sommer, H. H.
1951. The fat emulsion in milk from a chemical standpoint. The Milk Dealer 41 (1):58.

119. Spies, J. R.
1967. Determination of tryptophan in proteins. Anal. Chem., 39:1412.

120. Spies, J. R., and Chambers, D. C.
1948. Chemical determination of tryptophan. Anal. Chem., 20:30.

121. Storch, V.
1897. On the structure of "fat globules" in cow's milk. (Translated and communicated by H. Faber). Analyst, 22:197.

122. Sumner, J. B.
1944. A method for the colorimetric determination of phosphorus. Science, 100:413.

123. Swanson, A. M., and Sommer, H. H.
1940. Oxidized flavor in milk. I. Effect of the development of oxidized flavor on the iodine number of the phospholipid fraction of milk. J. Dairy Sci., 23:201.

124. Swope, F. C., and Brunner, J. R.
1965. Identification of ribonucleic acid in the fat-globule membrane. J. Dairy Sci., 48:1705.

125. Swope, F. C., and Brunner, J. R.
The fat globule membrane of cow's milk: A reassessment of isolation procedures and mineral composition. In review.

126. Swope, F. C., Brunner, J. R., and Vadehra, D. V.
1965. Riboflavin and its natural derivatives in the fat-globule membrane. J. Dairy Sci., 48:1707.

127. Tarassuk, N. P., and Frankel, E. N.
1957. The specificity of milk lipase. IV. Partition of the lipase system in milk. J. Dairy Sci., 40:418.

128. Tarassuk, N. P., and Koops, J.
1960. Inhibition of oxidized flavor in homogenized milk as related to the concentration of copper and phospholipids per unit of fat globule surface. J. Dairy Sci., 43:93.

129. Tarassuk, N. P., Koops, J., and Pette, J. W.
1959. The origin and development of trainy (fishy) flavor in washed cream and butter. I. Factors affecting the development of trainy flavor in washed cream. Neth. Milk Dairy J., 13:258.

130. Tayama, J.
1932. A simple method for the isolation of xanthine oxidase from milk. J. Biochem. (Japan), 17: 131.

131. Thompson, M. P., and Brunner, J. R.
1959. The carbohydrates of some glycoproteins of bovine milk. J. Dairy Sci., 42:369.

132. Thompson, M. P., Brunner, J. R., and Stine, C. M.
1959. Characteristics of high-melting triglyceride fractions from the fat-globule membrane and butter oil of bovine milk. J. Dairy Sci., 42:1651.

133. Thompson, M. P., Brunner, J. R., Stine, C. M., and Lindquist, K.
1961. Lipid components of the fat-globule membrane. J. Dairy Sci., 44:1589.

134. Titus, R. W., Sommer, H. H., and Hart, E. B.
1928. The nature of the protein surrounding the fat globules in milk. J. Biol. Chem., 76:237.

135. Tjepkema, R., and Richardson, T.
1966. Electrophoretic mobility of milk fat globules. III. Effect of diluent on mobility of fat globules and fat globule membrane. J. Dairy Sci., 49:1120.

136. Trautman, R.
1963. Computational methods of ultracentrifugation. In Ultracentrifugal Analysis in Theory and Experiments (J. W. Williams, ed.). Acad. Press., New York.

137. Vasić, J., and Man, J. M. de
1966a. The composition of the liquid phase of milk fat obtained by a pressure filteration method. Intern. Dairy Congr. XVII, Munich, C, 149.

138. Vasić, J., and Man, J. M. de
1966b. High melting glycerides and the milk fat globule membrane. Intern. Dairy Congr. XVII, Munich, C, 167.

139. Völtz, W.
1904. Untersuchungen über die Serumhüllen der Milchkügelchen. Pflug. Arch. Ges. Physiol., 102:373.

140. Wake, R. G., and Baldwin, R. L.
1961. Analysis of casein fractions by zone electrophoresis in concentrated urea. Biochim. Biophys. Acta. 47:225.

141. Warren, L.
1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234:1971.

142. Wathen, J. D., and Levy, R. S.
1966. Composition of the protein from dog lymph chylomicrons and dog serum high density lipoprotein. Biochemistry, 5:1099.

143. Weiss, L.
1963. The mammalian tissue cell surface. In The Structure and Function of the Membrane and Surfaces of Cells, Biochemical Society Symposium No 22 (D. J. Bell and J. K. Grant, eds.), Cambridge Univ. Press, Cambridge, England.

144. White, R. F., Eaton, H. D., and Patton, S.
1954. Relationship between fat globule surface area and carotenoid and vitamin A content of milk in successive portions of a milking. J. Dairy Sci., 37:147.

145. Whitney, R. McL.
1958. The minor proteins of bovine milk. J. Dairy Sci., 41:1303.

146. Wolf, D. P., and Dugan, L. R. Jr.
1964. Structure of high melting glycerides from the milk fat-globule membrane. J. Am. Oil Chemists' Soc., 41:139.

147. Wood, H. G., Gillespie, R., Seymour, J., Hansen, R. G., and Hardenbrook, H.
1958. Lactose synthesis. V. C^{14} in lactose, glycerol, and serine as indicators of the triose phosphate isomerase reaction and pentose cycle. J. Biol. Chem. 233:1271.

148. Zittle, C. A., DellaMonica, E. S., Custer, J. H., and Rudd, R. K.
1956. The fat globule membrane of milk: Alkaline phosphatase and xanthine oxidase in skim-milk and cream. J. Dairy Sci., 39:528.

APPENDIX

ABSTRACTS OF PUBLICATIONS

IDENTIFICATION OF RIBONUCLEIC ACID IN THE FAT GLOBULE MEMBRANE

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SUMMARY

Isolation and identification of a nucelic acid component, presumably of ribosomal origin, was obtained from fat globule membrane preparations. Membrane material was prepared by churning thrice-washed, raw cream separated from uncooled milk. Nucleic acid-rich fractions, obtained in yields of 5-10 mg per 40 liters of milk, were isolated from washed cream buttermilk using a sodium dodecyl sulfate-liquid phenol extraction procedure. Specific identification of nucleic acid was achieved by assay with crystalline pancreatic RNase. The TCA-soluble nucleotides were followed spectrophotometrically at 260 mμ.

It is suggested that the fat globule membrane contains small amounts of microsomal material.

J. Dairy Sci., 48:1705 (1965).

RIBOFLAVIN AND ITS NATURAL DERIVATIVES IN THE FAT GLOBULE MEMBRANE

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SUMMARY

A greenish-yellow color was observed in aqueous suspensions of fat globule membrane preparations. Liberation of this material from the membrane particulate is enhanced by addition of acid to pH 4.0 or the anionic dissociating agent sodium dodecyl sulfate. Freezing or lyophilization, or both, also dissociates this substance from the membrane.

Fluorometric assays made on the membrane preparations showed that the substance released possesses fluorescent characteristics similar to that observed in whey which, presumably, emanates from riboflavin. Of the total riboflavin found in the membrane, 92 to 95% exists as FAD, probably as the co-enzyme of xanthine oxidase. This compound is easily degraded by acid, dissociating agents and freeze-drying to riboflavin.

J. Dairy Sci., 48:1707 (1965).

APPARENT HOMOGENEITY OF LACTOPEROXIDASE IN GEL ELECTROPHORETOGRAMS

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SUMMARY

Lactoperoxidase (doner: H_2O_2 oxido-reductase, EC 1.11.1.7), a heme-containing protein which catalyzes the transfer of oxygen from peroxides to other substances, is one of the principal enzymes in cow's milk. Most of the available information on lactoperoxidase indicates that the enzyme exists in nature as isozymes or that process-induced alterations in the native protein might possibly account for the observation of other forms upon purification.

A lactoperoxidase-rich whey fraction was isolated from freshly drawn, uncooled cow's milk and submitted to zonal electrophoresis in a formic acid buffer, pH 3.8. The enzyme band was developed with a solution of guaiacol. Results of these studies suggest that lactoperoxidase exists as a single molecular species when isolated rapidly and that other forms may be caused by manipulations in isolation procedures.

J. Dairy Sci., 49:1279 (1966).

THE FAT GLOBULE MEMBRANE OF COW'S MILK: A REASSESSMENT OF ISOLATION PROCEDURES AND MINERAL COMPOSITION

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SUMMARY

Cream washed three times with three volumes of deionized water yielded fat globule membrane preparations essentially free of plasma components. Loss of loosely bound membrane material resulting from the washing process amounted to approximately 4% per washing. The loss was minimized when an isotonic sucrose-saline solution was employed as the washing medium. Membrane preparations obtained from three separate lots of Jersey, Brown Swiss milk by carefully controlled procedures amounted to 1.4 to 1.6 g/100 gr of fat. Whereas about 70% of the membrane was recovered from the buttermilk (aqueous membrane fraction) the remaining portion was closely associated with the butter granules (serum membrane fraction). The serum membrane contained considerably more lipids than the aqueous membrane fraction.

Molybdenum, iron and copper were the principal metallic components detected in membrane preparations. It is suggested that the Mo content is attributable exclusively to the presence of xanthine oxidase.

IN REVIEW

ISOLATION AND PARTIAL CHARACTERIZATION OF A GLYCOPROTEIN FROM THE FAT GLOBULE MEMBRANE

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SUMMARY

Fragmented fat globule membranes from thrice-washed cream were separated into 7,500S, 230S, and 35S pellets by ultracentrifugation. After lipid extraction the pellets were partially resolubilized with 1M KCl. A high carbohydrate-containing protein was found to reside primarily in the 35S fraction which accounted for approximately 9% of the pellet. The hexose of the protein was 7.8% and the total carbohydrate content was estimated to range between 15-19%. Some physical parameters of this glycoprotein were $S_{20,w}$ (11.6), $\overline{Mw}$ (306,500), $D_{20,w}$ (2.0) and μ (-3.6). The amino acid content showed relatively high amounts of lysine, threonine and glutamic acid as compared to the insoluble pellet proteins.

It is theorized that this protein may have a functional role in the creaming phenomenon of milk.

IN REVIEW