

# BIOCHEMICAL AND MORPHOLOGICAL COMPARISON OF PLASMA MEMBRANE AND MILK FAT GLOBULE MEMBRANE FROM BOVINE MAMMARY GLAND

T. W. KEENAN, D. JAMES MORRÉ, DIANE E. OLSON,  
W. N. YUNGHANS, and STUART PATTON

From the Departments of Animal Sciences, Biology and Botany and Plant Pathology, Purdue University, Lafayette, Indiana 47907, and The Lipids Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802

## ABSTRACT

Purified plasma membrane fractions from lactating bovine mammary glands and membranes of milk fat globules from the same source were similar in distribution and fatty acid composition of phospholipids. The sphingomyelin content of the phospholipid fraction of both membranes was higher than in these fractions from other cell components.  $\beta$ -carotene, a constituent characteristic of milk fat, was present in the lipid fraction of the plasma membrane. Cholesterol esters of plasma membrane were similar in fatty acid composition to those of milk fat globule membranes. Disc electrophoresis of either membrane preparation on polyacrylamide gels revealed a single major protein component characteristic of plasma membrane from other sources. Distinct morphological differences between plasma membrane and milk fat globule membranes were observed in both thin sections and in negatively stained material. Plasma membrane was vesicular in appearance while milk fat globule membranes had a platelike aspect. These observations are consistent with derivation of fat globule membrane from plasma membrane accompanied by structural rearrangement of membrane constituents.

## INTRODUCTION

The flow of fat through acinar cells of mammary tissue is very substantial. Small fat droplets form near the basal lamella and migrate toward the apical region of the cell as they mature (3). These globules range in size from 1 to 8  $\mu$  and average 3-4  $\mu$  in diameter. Such globules often approach the smaller dimensions of the cells in which they originate (28). How such large droplets are transported from the cells is a challenging question.

Bargmann and Knoop (3) concluded from electron microscope studies of lactating rat mam-

mary tissue that the milk fat droplets are progressively enveloped by the plasma membrane and ultimately pinched off into the alveolar lumen. Electron micrographs of bovine mammary tissue are also suggestive of this process (12). The Bargmann-Knoop hypothesis predicts direct derivation of the milk fat globule membrane (MFGM) from the plasma membrane. Patton and Fowkes (28) presented biochemical evidence in support of a role for the plasma membrane in enveloping the fat globule along with a rationale for the biophysics

of the process. These authors explained the envelopment of the droplet as being due to London-van der Waals attractive forces estimated to be several atmospheres at distances between membrane and droplet of less than 20 Å.

While it has been suggested that the MFGM consists of skim milk proteins adsorbed on the fat globule surface (19, 37), a number of findings are not consistent with this view. Fat globule membrane proteins differ in amino acid composition, in physical properties (15, 25), and immunologically (7, 24) from all but a minor protein component of skim milk. The lipids of the MFGM are largely polar lipids and sterols in contrast to those of the core fat, which consists principally of triglycerides (18, 36). Several enzymes generally associated with plasma membranes are found in MFGM (1, 9). Other authors propose that the fat globules in milk are surrounded by a continuous protein layer to which lipoprotein particles are adsorbed (13, 14, 22, 34). Brunner (5) has reviewed a number of lines of evidence which suggest that the MFGM differs at least morphologically from typical plasma membranes. Knoop, as cited by Brunner (5), did not detect a typical dark-light-dark pattern in the membrane surrounding the fat globule. Instead, she proposed that the cell membrane is quickly dissociated in the milk serum and that a new layer is formed for physical-chemical reasons. We offer here comparative biochemical and morphological evidence suggesting that MFGM is derived from the plasma membrane with accompanying structural rearrangement of the membrane constituents.

## MATERIALS AND METHODS

### *Tissue and Fat Globule Fractionation*

Milk and mammary tissue were obtained from several cows (Holstein) and one goat (Alpine-Toggenburg cross). Immediately before death, milk samples were drawn from the same gland from which mammary tissue was obtained. Milk samples were extracted to recover total lipids within 10 min after milking. Other portions of the milk were immediately cooled in ice and transported to the laboratory for MFGM isolation. Immediately postmortem, the udder was removed and mammary tissue recovered. Tissue was cut into thin slices and held in ice-cold 0.25 M sucrose during transportation to the laboratory (approximately 1.5 hr) where subsequent operations were performed at 2°C. Tissue was cut into approximately 1 cm<sup>3</sup> pieces that were washed with cold

0.25 M sucrose solution. Adipose and connective tissue were excluded insofar as possible.

Cow and goat mammary tissue (30 g wet weight) were placed in 150 ml of 0.25 M sucrose and homogenized with a Polytron 20 ST homogenizer (Kinematica, Lucerne, Switzerland) at lowest speed for 3–5 min. Debris, mitochondrial, microsomal, and postmicrosomal supernatant fractions were obtained from these homogenates according to Patton et al. (29). The floating lipid layer formed at various stages in the sedimentations was discarded.

Plasma membrane fractions were obtained from cow mammary tissue by a method based on the procedure of Neville (23) as modified by Emmelot et al. (11). Approximately 100 g of mammary tissue were homogenized in 1 mM potassium bicarbonate with the Polytron homogenizer. This homogenate was stirred at low speed for 5 min in 600 ml of bicarbonate solution, filtered through cheesecloth, and then centrifuged at 120 g for 10 min at –2°C to remove intact cells, debris, and nuclei. The supernatant was centrifuged at 1500 g for 10 min, and the resultant supernatant was carefully removed by aspiration and discarded. The friable upper portion of the pellet was resuspended in bicarbonate buffer. The tightly packed portion of the pellet was discarded. To eliminate gross mitochondrial contamination of the resultant preparation the above step was repeated 5–7 times. Plasma membrane was isolated from the final fraction by sucrose density gradient centrifugation as described (11). Plasma membrane was collected at the 1.16–1.18 density interface. Only those plasma membrane fractions judged to be greater than 80% pure by electron microscopic examination were subsequently analyzed.

Fat globules were recovered by centrifuging milk at 40,000 g for 1 hr at 5°C, washed with 0.9% saline, and subjected to one cycle of freezing and thawing (6, 18). Membranes were collected by suspending the ruptured globules in warm (35°C) 0.9% saline and centrifuging at 40,000 g for 1 hr at 5°C. The pellet was recovered and subjected to two resuspension-centrifugation cycles. Nascent fat droplets were recovered from tissue homogenates by collecting the lipid layer rising to the top during the first centrifugations used for recovery of plasma membrane. To free the droplets of adhering membranous fragments, they were washed with 1 mM bicarbonate, recovered by centrifugation at 40,000 g for 1 hr, subjected to one cycle of freeze-thawing, and again recovered from bicarbonate buffer as above.

### *Lipid Recovery and Analysis*

Milk and total tissue lipids were extracted as described previously (26). All solvents used were of reagent grade quality and were redistilled before use. Silicic acid column chromatography was used to

separate polar from neutral lipids of the milk and total tissue (16). Portions of the MFGM and cellular fractions were suspended in sucrose solution and extracted 3 or 4 times with several volumes of chloroform:methanol (2:1, v/v). The chloroform-rich layers were combined and evaporated to dryness *in vacuo* or under a stream of nitrogen at room temperature. Lipid residues were immediately weighed, redissolved in an accurately measured volume of chloroform (50  $\mu$ l/mg lipid), and stored at 2°C in sealed vials until analyzed.

Polar lipids were separated by thin-layer chromatography on 500- $\mu$  layers of silica gel HR (Brinkmann Instruments, Inc., Westbury, N.Y.). One-dimensional separations were accomplished in the solvent system chloroform:methanol:water (70:22:3, v/v/v). Two-dimensional separations were performed in the solvent systems described by Parsons and Patton (26). Spots were detected on plates by exposure to iodine vapors, recovered and analyzed for phosphorus by the procedures of Rouser et al. (32). Identity of the polar lipids of milk and mammary tissue has been established previously (10, 26). Additional evidence for the identity of lipid components was gained through the use of selective spray reagents and by cochromatography of lipid extracts with authentic reference compounds (Applied Science Labs., Inc., State College, Pa.). Identity of cerebroside was established by chromatography with authentic cerebroside and anthrone-positive reactions (31) of the material recovered from thin-layer plates. Neutral lipids were separated on 500- $\mu$  silica gel G plates in the solvent system petroleum ether:ethyl ether:acetic acid (90:10:1, v/v/v).  $\beta$ -carotene was separated from other components of the lipid extracts on silica gel G plates developed in hexane:benzene (4:1, v/v). In addition to thin-layer mobility, identity of  $\beta$ -carotene was established by comparing its ultraviolet spectrum (in hexane) to that of authentic  $\beta$ -carotene.

Fatty acid composition of the lipid classes recovered from thin-layer plates was determined by gas chromatography of the methyl esters. Lipid components were revealed by brief exposure to iodine vapors, recovered and transmethylated by previously reported methods (29). Visualization by exposure to iodine vapors was more sensitive and, as employed by us, yielded the same fatty acid composition as when the lipids were revealed by spraying with 2,7-dichlorofluorescein. Methyl esters were separated on a 2.4 m by 0.6 cm column packed with an ethylene glycol succinate polyester and operated isothermally at 170 or 190°C in an Aerograph (Varian Aerograph, Van Nuys, Calif.) Model 1520 gas chromatograph equipped with flame ionization detectors. Quantification of standard methyl ester mixtures (Supelco, Inc., Bellefonte, Pennsylvania) indicated that the major components (greater than 5%) were being analyzed with a relative error of less than 5%.

### Protein Analysis

Portions of the plasma membrane and MFGM pellets were dissolved in a phenol, acetic acid, water, and urea solution and subjected to polyacrylamide gel electrophoresis (35).

### Electron Microscopy

For negative staining, portions of the plasma membrane and MFGM preparations were mixed with equal volumes of deionized water and 2% phosphotungstic acid (neutralized to pH 6.8 with KOH), spread on carbon-stabilized, collodion-coated grids and air-dried. Portions of the same preparations were prepared for sectioning by fixation in 2% glutaraldehyde, postfixation in 1% OsO<sub>4</sub>, dehydration in an ethanol-acetone series, and embedding in an Epon-Araldite mixture (8). To insure adequate sampling, bands were collected from sucrose gradients by centrifugation by use of a swinging bucket rotor so that sedimentation was exactly perpendicular to the axis of rotation. To insure serial sections through all strata of sedimented material, sectors of the embedded pellets taken from near the pellet center were mounted in the microtome with the axis of sedimentation parallel to the plane of sectioning. Sections were then examined at intervals perpendicular to the axis of centrifugation. Specimens were observed and were photographed in a Philips EM 200 electron microscope.

### RESULTS

Plasma membrane fractions of high purity were obtained from lactating bovine mammary tissue. Mitochondria and collagen fibrils were the only consistent contaminants observed. The fractions isolated from bovine mammary gland were adjudged to represent plasma membrane on the basis of morphological criteria. Fragments consisted of large sheets of membranes that were interconnected through junctional complexes that are also present *in situ* (12, 20). In thin section, high magnification electron micrographs (not shown) show the typical triple-layered structure that clearly distinguishes these membranes from endoplasmic reticulum membranes, which show a globular appearance in thin section (4). The overall width of the membrane element was about 80-100 Å. Thinner membranes (50-60 Å), characteristic of the endoplasmic reticulum and nuclear envelope, were not observed in our preparations. Ribosomes were absent and, except for an occasional mitochondrion, the presence of other intracellular organelles could not be demonstrated in the plasma membrane preparations by electron

microscopy either in negative stain or in thin section. After negative staining the membranes appeared as large collapsed and distended sacs embedded in an amorphous film of phosphotungstate. The membrane surfaces showed a fine granular structure on the surface with smooth edges, features characteristic of plasma membrane isolated from other sources (4). These morphological markers, especially in negative contrast, allow one to readily distinguish the plasma membrane vesicles from other intracellular membranes and membrane fragments (4). The degree of contamination by cytoplasmic material contained within the vesicles was not assessed but was assumed to be minimal, for the reasons discussed by Benedetti and Emmelot (4). Approximately 12–15 mg of dry plasma membrane fraction was obtained from 100 g wet weight of mammary tissue. The major pigmented material was  $\beta$ -carotene as identified by its identical mobility with authentic  $\beta$ -carotene on thin-layer plates. The ultraviolet absorption spectra from 300 to 600  $m\mu$  for both sample and reference material were identical with a single maximum at 440  $m\mu$  and a shoulder at 465  $m\mu$ . Milk fat globule membranes, released and purified from isolated milk fat globules, appeared homogeneous but electron microscopic examination showed them to contain small quantities of microsome-like membrane fragments. The yield of MFGM was 12–16 mg dry weight/100 ml milk.

Two-dimensional thin-layer chromatography of membrane lipids in solvent systems that separate all known polar lipids of milk and mammary tissue revealed the same qualitative distribution in both MFGM and plasma membrane (Fig. 1). Both preparations appeared to contain the same relative proportions of cerebrosides. Mono- and dihexose cerebrosides were not observed in the lipids of cell fractions from the goat and appeared to be concentrated in the plasma membrane from bovine preparations. Detailed analyses of the phospholipid distribution of two plasma membrane fractions and the corresponding MFGM fractions revealed a remarkable similarity in distribution of all five components (Table I). Other mammary tissue cell fractions did not show this distribution (Table II; 28).

Comparing polar lipids from cell components, milk and total mammary tissue from the cow (Table II), sphingomyelin (relative to total lipid phosphorus) was higher in milk than the other cell

fractions analyzed (Table II). With goat mammary tissue, important differences in phospholipid distribution were noted when comparing milk and tissue fractions (Fig. 2). In contrast, the relative sphingomyelin content of plasma membrane was high and sufficient to account for the relative amounts of sphingomyelin observed in milk (Table II), if direct origin of MFGM from plasma membrane is assumed.

Disc electrophoresis of MFGM and plasma membrane total protein on polyacrylamide gels revealed an identical major protein component in each fraction (Fig. 3). The only demonstrable difference in the two patterns was the presence of one additional minor band in the MFGM fraction (identified by the arrow in Fig. 3).

All phospholipids were observed to have the same principal (greater than 2%) fatty acids when comparing plasma membrane and MFGM (Fig. 4). Although not drastic, some variation in fatty acid composition was observed. The plasma membrane fraction analyzed was estimated to contain about 15% mitochondria whereas microsome-like material contaminated the MFGM preparations. Comparisons of the fatty acid composition of the cholesterol esters showed a much closer similarity between plasma membrane and MFGM (Table III). Palmitate and oleate were the only acids that differed appreciably between the two fractions. Even the comparative levels of the minor acids showed this homology. Cholesterol ester fatty acids from other cell fractions were not similar to those of MFGM.

Nascent fat droplets (the cell cream which rises to the top on the first centrifugations) were observed to contain small but significant amounts of phospholipid. When separated from the bulk of the neutral lipid on silicic acid columns, polar lipids constituted about 0.5–1% of the total fat. The phospholipid distribution of these droplets was different from that of the MFGM and plasma membrane (Table I). Nearly half of the lipid phosphorus of nascent fat was accounted for as phosphatidyl- and lysophosphatidyl choline. Core fat (the oil recovered on lysis of milk fat globules) was extremely poor in polar lipids (less than 0.2% of the total).

Electron microscopy of negatively stained plasma membrane, MFGM and intact milk fat globules revealed distinct morphological differences (Figs. 5–10). Fat globule membranes exhibited a platelike structure (Figs. 7 and 8),

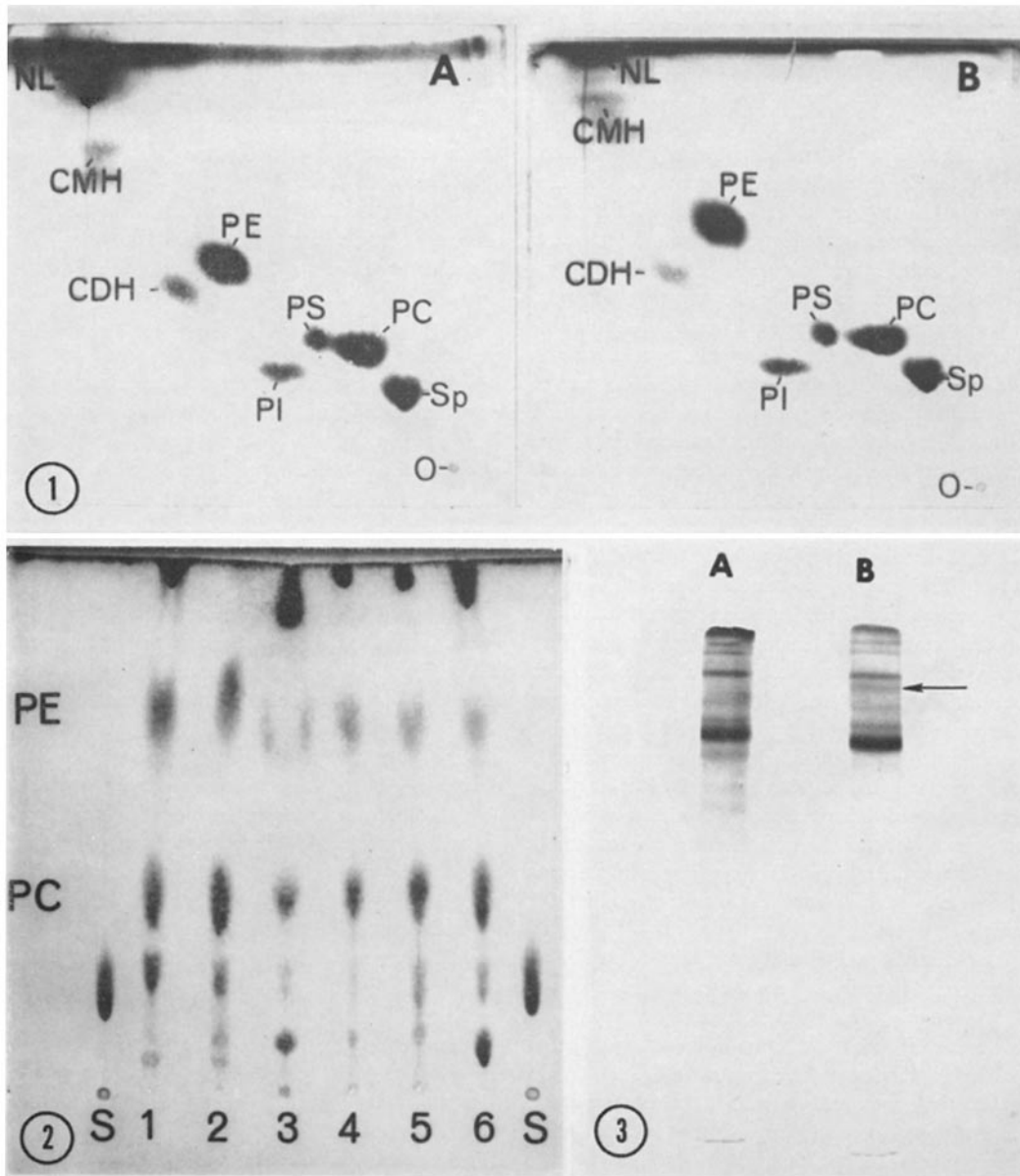


FIGURE 1 Two-dimensional thin-layer chromatograms of plasma membrane (A) and milk fat globule membrane (B) total lipids. *O*, origin; *Sp*, sphingomyelin; *PC*, phosphatidyl choline; *PS*, phosphatidyl serine; *PI*, phosphatidyl inositol; *PE*, phosphatidyl ethanolamine; *CDH*, cerebroside dihexoside; *CMH*, cerebroside monohexoside; *NL*, neutral lipid. Visualized by charring with chromic acid. Approximately equal quantities of the lipid from each fraction were applied to the plate.

FIGURE 2 Thin-layer chromatogram of the total lipids of milk and total tissue and polar lipids of cell fractions of lactating goat mammary tissue. *S*, reference sphingomyelin; *1*, milk; *2*, total tissue; *3*, debris; *4*, mitochondria; *5*, microsomes; *6*, supernatant. *PC*, phosphatidyl choline, *PE*, phosphatidyl ethanolamine. Material at solvent front is neutral lipid. Visualized by charring with chromic acid. Approximately equal quantities of lipid from each fraction were applied to the plate.

FIGURE 3 Polyacrylamide gel electrophoretic patterns of total protein from plasma membrane (A) and milk fat globule membrane (B).

TABLE I  
*Phospholipid Distribution in the Plasma Membrane, Milk Fat Globule Membrane, and Nascent Fat from the Lactating Cow\**

Lipid component	Cow A			Cow B		
	Plasma membrane	Milk fat globule membrane	Nascent fat	Plasma membrane	Milk fat globule membrane	Nascent fat
Sphingomyelin	22.0 ± 4.1	21.4 ± 2.9	10.1 ± 3.0	27.0 ± 2.4	22.4 ± 2.5	10.3 ± 1.5
Phosphatidyl choline	27.0 ± 4.2	25.7 ± 3.0	38.8 ± 1.7	30.8 ± 3.8	31.7 ± 1.3	42.9 ± 3.9
Phosphatidyl serine	8.4 ± 0.4	14.0 ± 2.3	11.5 ± 1.0	8.5 ± 0.8	9.1 ± 0.6	6.2 ± 2.0
Phosphatidyl inositol	13.2 ± 3.4	11.1 ± 2.2	9.7 ± 2.6	12.3 ± 2.6	10.3 ± 0.8	8.2 ± 1.1
Phosphatidyl ethanolamine	29.4 ± 1.9	27.8 ± 1.3	22.0 ± 0.8	21.3 ± 2.8	27.2 ± 0.4	21.7 ± 3.6
Lysophosphatidyl choline	—	—	7.9 ± 2.9	—	—	10.7 ± 3.2

\* Values are means plus and minus standard deviations, n = 3. Expressed as % of total lipid phosphorus.

whereas plasma membranes appeared as vesicular sheets (Figs. 5 and 6). The platelike aspect of MFGM was especially evident in negatively stained preparations of intact fat globules where the membrane appeared to be partially pulled

away from the fat during staining (Fig. 10). In thin sections of MFGM, short rodlike profiles were observed (Fig. 8). Thin sections of plasma membrane contained large and small vesicles with undulating profiles (Fig. 6). The vesicular nature of the isolated plasma membrane fraction was evident in both negatively stained (Fig. 5) and sectioned material (Fig. 6), but was most clearly demonstrated by occasional preparations where membrane blebbing resulted in the formation of many small vesicles still attached to a large fragment (Fig. 9).

#### DISCUSSION

Detailed analyses of phospholipid constituents when comparing plasma membrane and milk fat globule membrane (MFGM) of bovine mammary gland reveal a similar distribution of all components. This is strongly suggestive of direct plasma membrane origin of MFGM. However, when comparing plasma membrane and MFGM with other cell fractions from mammary gland, major differences are evident. The sphingomyelin contents of milk, MFGM, and plasma membrane are

TABLE II  
Sphingomyelin Content of Milk and Mammary Tissue Fractions\*

Fraction	Sphingomyelin	
	%	
Milk‡	28.4	
Total tissue	10.2	
Debris	8.5	
Mitochondria	6.6	
Microsomes	12.6	
Supernatant	12.9	
Milk§	18.0	23.7
Plasma membrane	18.9	20.9

\* Expressed as % of total lipid phosphorus. Values are averages of duplicate analyses.

‡ Samples derived from a lactating cow.

§ Samples derived from two lactating cows.

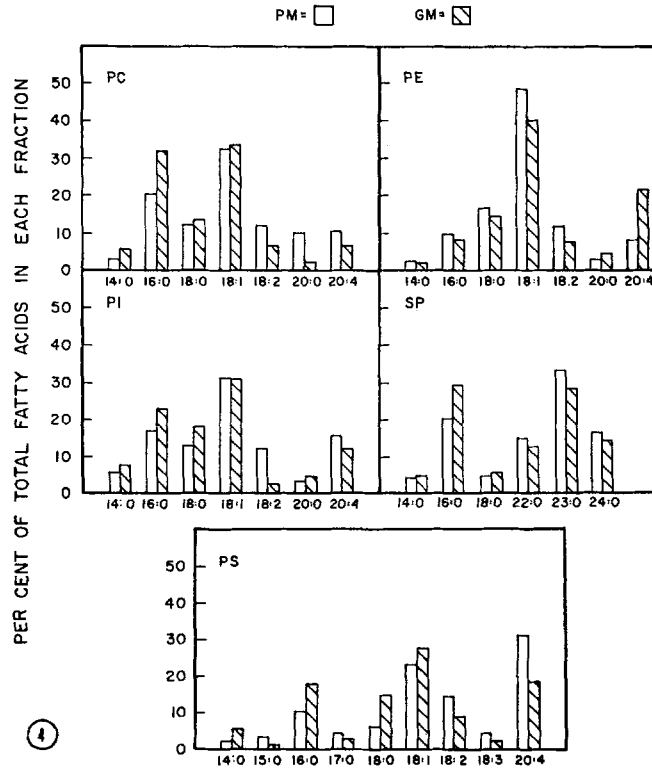


FIGURE 4 Major fatty acids of individual phospholipids from plasma membrane (PM) and milk fat globule membrane (GM). Number before colon gives the number of carbons; number after colon gives the number of double bonds. Other abbreviations as in Fig. 1.

TABLE III  
*Fatty Acid Composition of the Cholesterol Ester Fraction of Plasma Membrane and Milk Fat Globule Membrane from the Lactating Cow*

Fatty acid*	Plasma membrane	Milk fat globule membrane
	weight %	weight %
10:0	3.9	5.1
10:1	0.3	0.4
11:0	2.4	1.8
12:0	3.2	3.8
12:1	0.7	0.4
13:0	0.3	0.2
13:1	1.2	1.3
14:0	9.1	9.7
14:1	2.1	1.7
Unknown	0.3	0.3
15:0	1.8	2.4
15:1	3.1	3.2
16:0	21.3	24.9
16:1	10.4	9.6
17:0	1.3	1.2
17:1	2.9	1.7
18:0	4.0	5.3
18:1	30.0	25.8
18:2	1.6	1.2

\* Number before the colon gives the number of carbon atoms; number after the colon gives the number of double bonds.

nearly equal and at least two times that of the total tissue and higher than that of other cell fractions on a lipid phosphorus basis. The distribution of the major phospholipids in total milk and the MFGM is virtually identical (18, 21, 26, 27) but different from that of other cell fractions. The sphingomyelin content of milk is largely derived from MFGM, and its content in plasma membrane is sufficiently high to account for that present in MFGM. On analysis of Golgi apparatus, endoplasmic reticulum, and plasma membrane fractions from rat liver (T. Keenan and D. Morr , unpublished observations), only plasma membrane was found to have the uniquely high sphingomyelin content characteristic of MFGM and plasma membrane from the bovine. Although the close correlation in sphingomyelin content is most striking, the distribution of other phospholipids of rat liver plasma membrane is similar to that of MFGM and milk (18, 21, 26).

Important differences between MFGM and plasma membrane were encountered, however, in comparisons of fatty acid composition of the

phospholipids. Exchange during homogenization and centrifugation (41), and contamination by other cell components may contribute to these differences. Exchange between fatty acids of lipids of the MFGM and those of the underlying core fat is likely, and there is no reason to suppose that plasma membrane lipids are uniformly distributed within the membrane. For example, fatty acids of the apical portion of the plasma membrane specifically involved in the formation of MFGM might differ from those of the remainder of the membrane by virtue of its more rapid turnover in bulk. In any event, the observed differences in fatty acids were not great enough to preclude origin of MFGM from plasma membrane.

Perhaps a more critical comparison is the cholesterol ester fatty acid composition, since these constituents have been found to replenish their fatty acids only very slowly, if at all, in milk after secretion (T. W. Keenan and S. Patton, Cholesterol esters of milk and mammary tissue. In preparation). This comparison, when made in some detail, revealed a remarkable homology of cholesterol ester fatty acids between plasma membrane and MFGM.

Finally, the disc gel electrophoretic patterns of plasma membrane and MFGM are similar, each having a single major protein component. A similarly migrating major protein component is also characteristic of plasma membrane from rat liver. This band is not observed as a major component in patterns obtained for Golgi apparatus, endoplasmic reticulum, and mitochondrial preparations from rat liver and carried out in a manner identical to that used for the preparations reported here (W. Yunghans, T. W. Keenan, and D. Morr , in preparation).

The oil recovered on lysis of the milk fat globules (core fat of the globule interiors) is extremely poor in polar lipids. This is in contrast to the nascent fat droplets (cell cream) that contain small but significant amounts of phospholipids. Nascent fat represents droplets in all stages of synthesis, whereas core fat is predominantly completed product. For example, nascent fat is rich in phosphatidyl- and lysophosphatidyl choline, compounds that have been suggested to be intermediates in the formation of milk triglycerides (30). Electron microscopic examination has shown that the nascent fat droplets do not possess a discernible membrane (2, 3). However, there is no evidence to rule out the possibility that their sur-



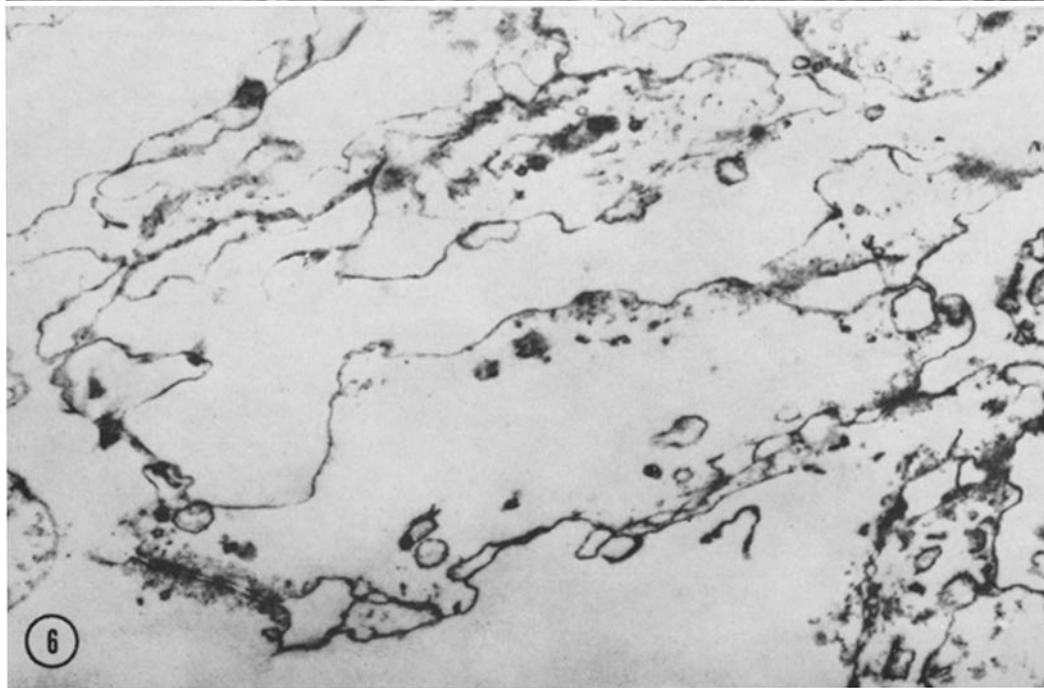
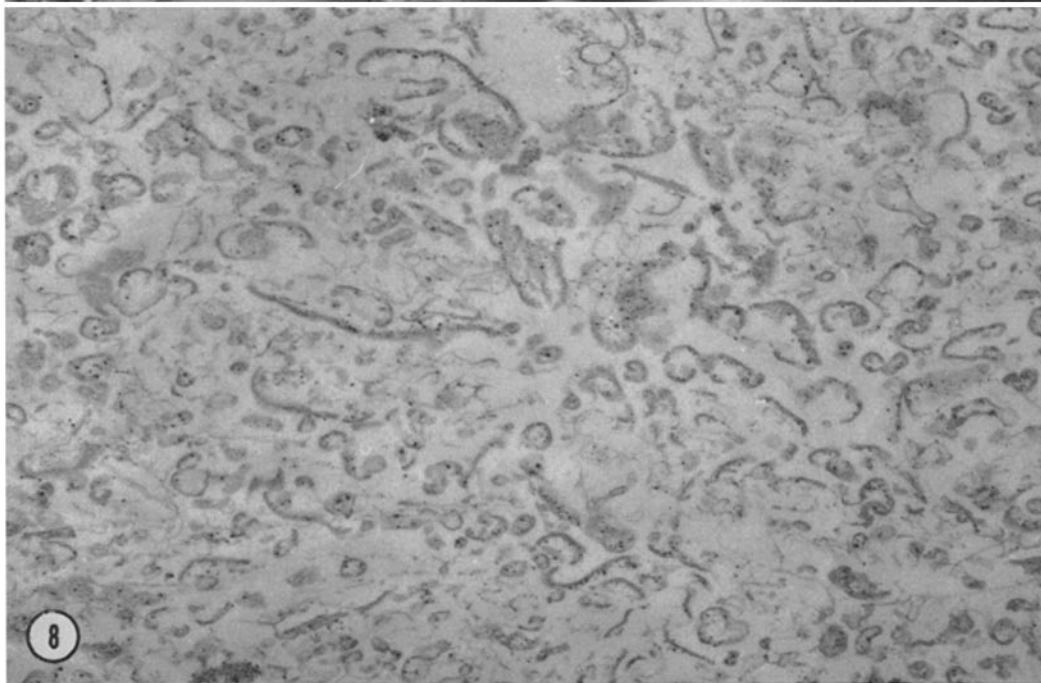
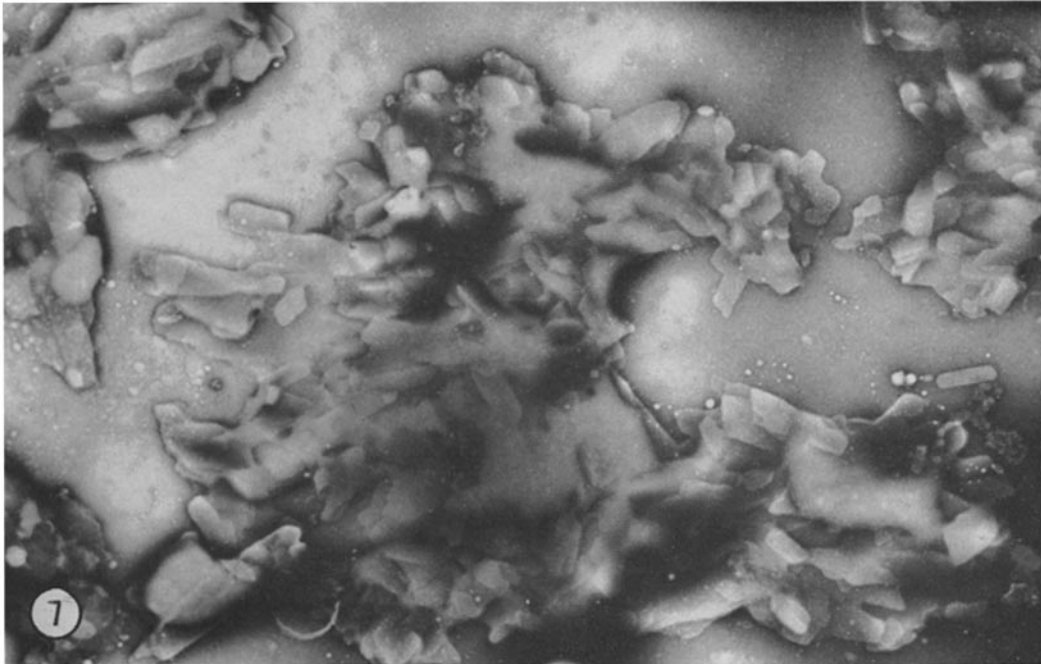


FIGURE 5 Plasma membrane-rich fraction from lactating bovine mammary tissue in negative contrast after staining with phosphotungstic acid (PTA). The collapsed vesicles are embedded in a thin, amorphous film of stain and show a fine granular or fibrous texture with smooth edges in surface view. Vesicle margins appear smooth and electron transparent.  $\times 26,000$ .

FIGURE 6 Plasma membrane-rich fraction from lactating bovine mammary gland in thin section. Preparations contained vesicles of many sizes or long undulating membrane profiles with occasional desmosomes and junctional complexes to identify them as plasma membrane fragments. Material, the composition of which is unknown, is seen adhering to junctional complexes. Tangential cuts through large membrane fragments cause a loss of clear membrane images and give the appearance of "smudged profiles."  $\times 40,000$ .



**FIGURE 7** Milk fat globule membrane fraction in negative contrast after staining with PTA. The membranes have an irregular surface but appear smooth with no evidence of a granular or fibrous texture. Much of the surface appears to repel the stain. The margins end abruptly lending a platelike aspect to individual fragments (compare with Fig. 5).  $\times 21,000$ .

**FIGURE 8** Milk fat globule membrane fraction in thin section. The bulk of the preparation consists of short, rodlike profiles with free ends in contrast to the closed vesicular profiles characteristic of plasma membrane (compare with Fig. 6). The membrane binds stain in a manner that causes the formation of small electron-opaque grains that resist washing of the section.  $\times 22,000$ .

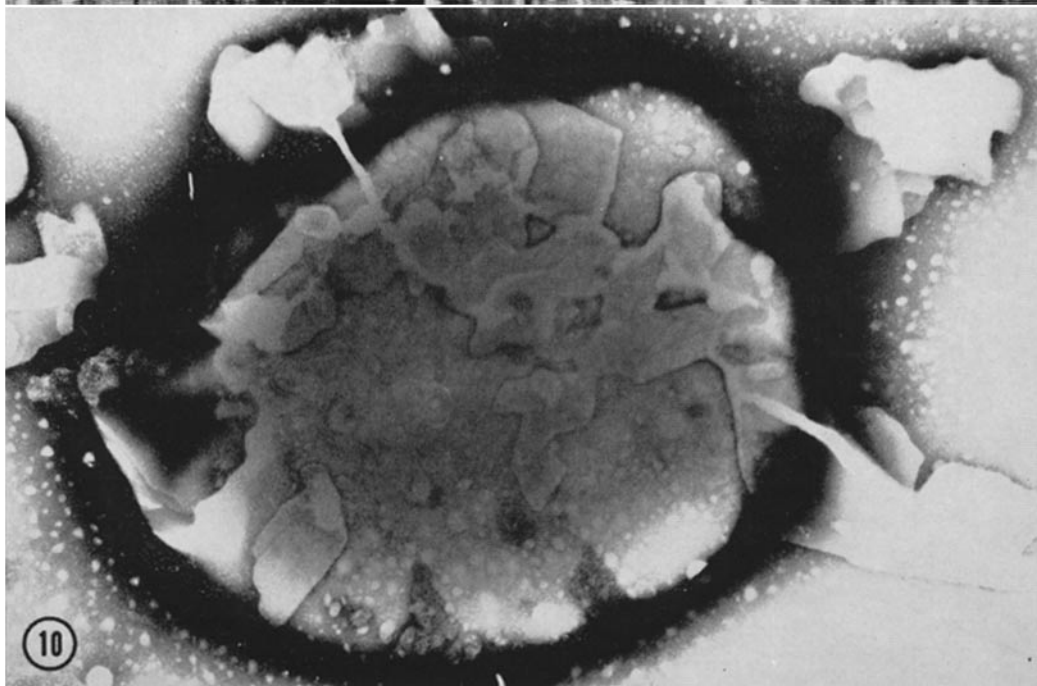
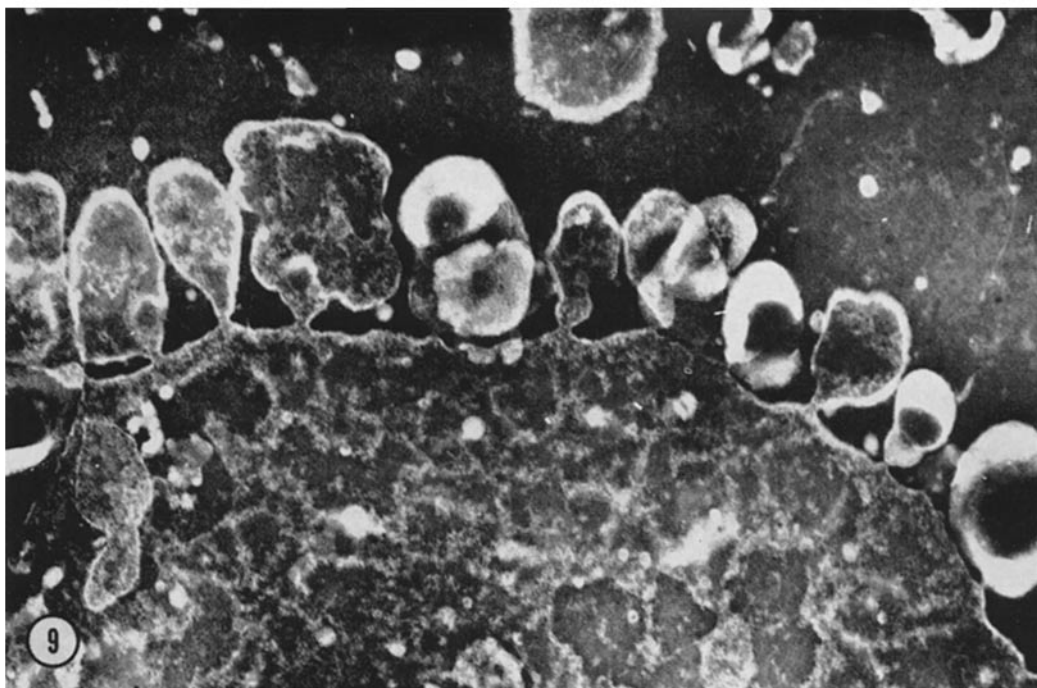


FIGURE 9 Negative contrast of a plasma membrane fragment from lactating bovine mammary gland after staining with PTA. Many small vesicles are shown attached to a larger fragment.  $\times 48,000$ .

FIGURE 10 Negative contrast of an intact milk fat globule after staining with PTA. The platelike aspect and tendency of the milk fat globule membrane to resist vesiculation is apparent where portions of the membrane have pulled away from the fat during staining.  $\times 44,000$ .

faces are bounded by polar lipid and/or protein layers (3). Our findings of phospholipids in the nascent fat droplet fraction support this suggestion.

Among the components absent from the nascent fat but plainly evident in milk fat are the carotenoids. Lipids of the plasma membrane are a deep orange-yellow color, while lipids of the cell cream (nascent fat) are nearly colorless. White et al. (40) found that  $\beta$ -carotene, the major carotenoid of milk, is distributed in milk fat globules in direct relation to surface area. In the present study,  $\beta$ -carotene was the major pigmented material encountered in bovine plasma membrane, and the plasma membrane origin of MFGM would explain the origin of  $\beta$ -carotene in milk.

The qualitative and quantitative biochemical similarities of plasma membrane and MFGM are not reflected in their morphologies as revealed by the electron microscope. Electron micrographs of the isolated cell fractions presented here and in vivo studies (2, 3, 12, 17, 20, 33, 38, 39) show their morphologies to be different.

The early observation of Bargmann and Knoop (3) that milk fat droplets are progressively enveloped by the plasma membrane and ultimately pinched off into the alveolar lumen has since been confirmed by a number of investigators (2, 12, 20, 38, 39). Electron micrographs presented by these authors indicate that the structural integrity of the plasma membrane is maintained during envelopment of the fat droplet. Although there are conflicting views as to whether small amounts of cytoplasm are constricted along with the droplet, it is clear that large amounts of cytoplasm are not released with lipid droplets (2, 3, 20). The mechanism of lipid secretion is highly efficient in that it does not permit mitochondria to pass into the gland lumen (29). Electron microscope-radioautographic studies of Stein and Stein (33) show that small fragments of the rough endoplasmic reticulum, which appears to be the site of elaboration of lipid droplets, adhere to the fat globule after secretion. These fragments may be the source of the microsome-like membrane fragments observed in MFGM preparations in the present study.

Whereas other membranes (including plasma membrane) tend to form vesicles when disrupted during tissue homogenization, the MFGM shows

no tendency to vesiculate. MFGM is present in the isolated preparations as relatively rigid plates, whereas the bovine plasma membrane exhibits the highly deformable and vesicular morphology characteristic of plasma membranes from other sources (4). Differences in staining and thickness of the membrane also exist for the isolated preparations but will require a more detailed study, along with more knowledge of lipid:protein ratios, to assess their significance. At present, the differences in gross morphology are sufficient to emphasize the point that if MFGM is derived largely from plasma membrane without quantitative or qualitative changes in membrane constituents, the process must at least involve a rearrangement of constituents within the membrane.

Although our results are not in themselves proof, when they are taken together with information from in vivo studies (2, 3, 12, 20, 38, 39) there is little doubt that MFGM originates directly from the plasma membrane. Direct contribution of the phospholipids, cerebrosides, cholesterol esters,  $\beta$ -carotene, and major protein constituents of MFGM by plasma membrane is indicated. Structural rearrangement or changes in the lipid:protein ratio would then be necessary to account for the morphological differences between MFGM and plasma membrane. From a purely physical standpoint, it is reasonable to assume that the plasma membrane, which is relatively stable in the predominantly aqueous environment of the cytoplasm, would undergo a rearrangement when it comes in contact with the less polar, more hydrophobic fat droplet. The London-van der Waals attractive forces between the droplet and membrane appear to be sufficient to effect the reorientation of membrane constituents (28), but a more precise understanding of the nature of the reorientation must await further study.

The assistance of Prof. E. L. Benedetti in preparing this manuscript is gratefully acknowledged.

This work was supported in part by the National Science Foundation (GB 03044 and 7078). Purdue University AES Journal Paper No. 3669.

Received for publication 23 May 1969, and in revised form 28 August 1969.

#### REFERENCES

1. BAILE, M. J., and R. K. MORTON. 1958. Comparative properties of microsomes from cow's milk and mammary gland. I. Enzymatic activities. II. Chemical composition. *Biochem. J.* 69:35, 44.
2. BARGMANN, W., K. FLEISCHHAUER, and A.

- KNOOP. 1961. Über die morphologie der milchsekretion. II. Zugleich eine kritik am schema der sekretionsmorphologie. *Z. Zellforsch. Mikrosk. Anat.* 53:545.
3. BARGMANN, W., and A. KNOOP. 1959. Über die morphologie der milchsekretion. I. Licht-und elektronenmikroskopische studien an der milchdrüse der ratte. *Z. Zellforsch. Mikrosk. Anat.* 49:334.
  4. BENEDETTI, E. L., and P. EMMELOT. 1966. Structure and function of plasma membranes isolated from liver. In *Ultrastructure in Biological Systems*. A. J. Dalton and F. Haguenu, editors. Academic Press Inc., New York. 4:33.
  5. BRUNNER, J. R. 1966. Physical equilibria in milk: Fat. In *Fundamentals of Dairy Chemistry*. B. H. Webb and A. H. Johnson, editors. Avi, Westport, Conn. 403.
  6. COLE, L. J. N., D. KLUEPFEL, and C. V. LUSENA. 1959. Freezing damage to bovine cream indicated by release of enzymes. *Can. J. Biochem. Physiol.* 37:821.
  7. COULSON, E. J., and R. H. JACKSON. 1962. The mucoprotein of the fat/plasma interface of cow's milk. II. Immunochemical characterization. *Arch. Biochem. Biophys.* 97:378.
  8. CUNNINGHAM, W. P., D. J. MORRÉ, and H. H. MOLLENHAUER. 1966. Structure of isolated plant Golgi apparatus revealed by negative staining. *J. Cell Biol.* 28:169.
  9. DOWBEN, R. M., J. R. BRUNNER, and D. E. PHILPOTT. 1967. Studies on milk fat globule membranes. *Biochim. Biophys. Acta.* 135:1.
  10. DUTHIE, A. H., and S. PATTON. 1965. Purification of phospholipids recovered from thin-layer chromatograms for infrared spectral analysis. *J. Lipid Res.* 6:320.
  11. EMMELOT, P., C. J. BOS, E. L. BENEDETTI, and PH. RUMKE. 1964. Studies on plasma membranes. I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. *Biochim. Biophys. Acta.* 90:126.
  12. FELDMAN, J. D. 1961. Fine structure of the cow's udder during gestation and lactation. *Lab. Invest.* 10:238.
  13. HAYASHI, S., D. R. ERICKSON, and L. M. SMITH. 1965. Membranous material of bovine milk fat globules. II. Some physical and enzymic properties of the deoxycholate-released lipoproteins. *Biochemistry.* 4:2557.
  14. HAYASHI, S., and L. M. SMITH. 1965. Membranous material of bovine milk fat globules. I. Comparison of membranous fractions released by deoxycholate and by churning. *Biochemistry.* 4:2550.
  15. HERALD, C. T., and J. R. BRUNNER. 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.
  16. HIRSCH, J., and E. H. AHRENS. 1958. The separation of complex lipide mixtures by the use of silicic acid chromatography. *J. Biol. Chem.* 233:311.
  17. HOLLMANN, K.-H. 1959. L'ultrastructure de la glande mammaire normale de la souris en lactation. Etude au microscope electronique. *J. Ultrastruct. Res.* 2:423.
  18. HUANG, T. C., and A. KUKSIS. 1967. A comparative study of the lipids of globule membrane and fat core and of the milk serum of cows. *Lipids.* 2:453.
  19. KING, N. 1955. *The Milk Fat Globule Membrane*. Commonwealth Agricultural Bureaux, Farnham, Royal, England.
  20. KUROSUMI, K., Y. KOBAYASHI, and N. BABA. 1968. The fine structure of mammary glands of lactating rats, with special reference to the apocrine secretion. *Exp. Cell Res.* 50:177.
  21. MORRISON, W. R. 1968. The distribution of phospholipids in some mammalian milks. *Lipids.* 3:101.
  22. MORTON, R. K. 1954. The lipoprotein particles in cow's milk. *Biochem. J.* 57:231.
  23. NEVILLE, D. M. 1960. The isolation of a cell membrane fraction from rat liver. *J. Biophys. Biochem. Cytol.* 8:413.
  24. PALMER, L. S., and J. J. H. LEWIS. 1933. The serologic reactions of a new protein from milk. *Arch. Pathol.* 16:303.
  25. PALMER, L. S., and E. SAMUELSSON. 1924. The nature of the substances adsorbed on the surface of the fat globules in cow's milk. *Proc. Soc. Exp. Biol. Med.* 21:537.
  26. PARSONS, J. G., and S. PATTON. 1967. Two dimensional thin-layer chromatography of the polar lipids from milk and mammary tissue. *J. Lipid Res.* 8:696.
  27. PATTON, S., A. DURDAN, and R. D. MCCARTHY. 1964. Structure and synthesis of milk fat. VI. Unity of the phospholipids in milk. *J. Dairy Sci.* 47:489.
  28. PATTON, S., and F. M. FOWKES. 1967. The role of the plasma membrane in the secretion of milk fat. *J. Theor. Biol.* 15:274.
  29. PATTON, S., L. F. HOOD, and J. S. PATTON. 1969. Negligible release of cardiolipin during milk secretion by the ruminant. *J. Lipid Res.* 10:260.
  30. PATTON, S., R. O. MUMMA, and R. D. MCCARTHY. 1966. Pathways in biosynthesis of milk fat. *J. Dairy Sci.* 49:737.
  31. RADIN, N. S., F. B. LAVIN, and J. R. BROWN. 1955. Determination of cerebrosides. *J. Biol. Chem.* 217:789.
  32. ROUSER, G., A. N. SIAKOTOS, and S. FLEISCHER. 1966. Quantitative analysis of phospholipids by

- thin-layer chromatography and phosphorus analysis of spots. *Lipids*. **1**:85.
33. STEIN, O., and Y. STEIN. 1967. Lipid synthesis, intracellular transport, and secretion. II. Electron microscope radioautographic study of the mouse lactating mammary gland. *J. Cell Biol.* **34**:251.
  34. SWOPE, F. C., and J. R. BRUNNER. 1965. Identification of ribonucleic acid in the fat globule membrane. *J. Dairy Sci.* **48**:1705.
  35. TAKAYAMA, K., D. H. MACLENNAN, A. TZAGOLOFF, and C. D. STONER. 1966. Studies on the electron transfer system. LXVII. Polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. *Arch. Biochem. Biophys.* **114**:223.
  36. THOMPSON, M. P., J. R. BRUNNER, C. M. STINE, and K. LINDQUIST. 1961. Lipid components of the fat globule membrane. *J. Dairy Sci.* **44**: 1589.
  37. TITUS, R. W., H. H. SOMMER, and E. B. HART. 1928. The nature of the protein surrounding the fat globules in milk. *J. Biol. Chem.* **76**:237.
  38. WELLINGS, S. R., K. B. DEOME, and D. R. PITELKA. 1960. Electron microscopy of milk secretion in the mammary gland of the C3H/CRgl mouse. I. Cytomorphology of the prelactating and the lactating gland. *J. Nat. Cancer Inst.* **25**:393.
  39. WELLINGS, S. R., B. W. GRUNBAUM, and K. B. DEOME. 1960. Electron microscopy of milk secretion in the mammary gland of the C3H/CRgl mouse. II. Identification of fat and protein particles in milk and in tissue. *J. Nat. Cancer Inst.* **25**:423.
  40. WHITE, R. F., H. D. EATON, and S. PATTON. 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.
  14. WIRTZ, K. W. A., and D. B. ZILVERSMIT. 1968. Exchange of phospholipids between liver mitochondria and microsomes in vitro. *J. Biol. Chem.* **243**:3596.