LIPIDS IN FRACTIONS OF THE FAT GLOBULE MEMBRANE OF COWS' MILK

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Leo W. Quirk

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THESIS

ROOM USE ONLY

ABSTRACT

LIPIDS IN FRACTIONS OF THE FAT GLOBULE MEMBRANE OF COWS' MILK

by Leo W. Quirk

The fat globule membrane of cows' milk is the lipid-proteinaceous material lying on the surface of the fat globule.

The objectives of this study were as follows:

(1) to isolate an aqueous suspension of fat globule membrane of cows' milk;

(2) to fractionate the aqueous suspension into three pellets by the technique of differential sedimentation; and

(3) to determine the distribution of total lipids, neutral lipids, polar lipids, and major lipid classes (e.g., monoglycerides) among the three pellets.

The aqueous suspension of the fat globule membrane was obtained from fresh, uncooled, whole milk. The milk was separated into skim-milk and cream. The cream was washed with water, adjusted to 8°C, and churned. The churned mass was warmed until the butter melted completely to liquid. The liquid mixture was allowed to stand in a separatory funnel until two phases formed. The lower phase, an aqueous suspension of the fat globule membrane, was separated from the upper phase.

By application of the technique of differential sedimentation in the ultracentrifuge the membrane suspension was fractionated into three pellets. Calculations gave the approximate minimum sedimentation coefficients of the three pellets as 7,500S, 230S, and 35S.

Analyses of the lipids in the pellets were performed on two separate preparations. The lipids in the aqueous pellet suspensions of preparation No. 1 were extracted with chloroform-methanol, 2:1 (v/v). The extracts were washed with an aqueous solution of potassium chloride, and the lipids were separated into polar lipids and neutral lipids by silicic acid column method of Ways and Hanahan[a] (1964). The neutral lipids were separated into major classes by chromatography on thin layers of silica gel G. Each major class was eluted separately from the adsorbent and weighed.

The aqueous pellet suspensions of preparation No. 2 were dried by lyophilization. Extraction of the lipids of the lyophilized pellets and washing of the extracts were accomplished by the methods used for preparation No. 1. The pellet lipids were mixed in chloroform with heat-activated silicic acid. After the neutral lipids were eluted from the silicic acid with chloroform, the polar lipids were eluted with methanol and were then separated into major classes on thin layers of silica gel G. Each major class was eluted separately from the adsorbent. Phosphorus analysis of each eluate provided the relative amounts of the major classes of phospholipids in each pellet.

The conclusions were as follows:

(1) the total lipids of the 230S and 35S pellets contained higher percentages of polar lipids than did the total lipids of the 7,500S pellet;

(2) the neutral lipids of the 230S and 35S pellets contained higher

percentages of cholesterol than did the neutral lipids of the 7,500S pellet;

(3) the neutral lipids of the 35S pellet contained higher percentages of monoglycerides, diglycerides, and fatty acids and a lower percentage of triglycerides than did the neutral lipids of the 7,500S and 230S pellets;

(4) the polar lipids of the 35S pellet contained a higher percentage of sphingomyelin and a lower percentage of lecithin than did the polar lipids of the 7,500S and 230S pellets; and

(5) the phospholipids of whole milk contain more cephalin and less lecithin on a percentage basis than do the phospholipids of the isolated membrane fraction (the three pellets).

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[a] Peter Ways and Donald J. Hanahan, "Characterization and Quantification of Red Cell Lipids in Normal Man," Journal of Lipid Research, 5: 318-328 (July, 1964).

LIPIDS IN FRACTIONS OF THE FAT GLOBULE MEMBRANE OF COWS' MILK

By

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INTRODUCTION

The fat globule membrane of cows' milk is the lipid-proteinaceous material lying on the surface of the fat globule. The discovery of the fat globule membrane and the post-discovery research concerned with the membrane are described in the REVIEW OF THE LITERATURE.

The objectives of this study were as follows:

(1) to isolate an aqueous suspension of the fat globule membrane of cows' milk;

(2) to fractionate the aqueous suspension into three pellets by the technique of differential sedimentation; and

(3) to determine the distribution of total lipids, neutral lipids, polar lipids, and major lipid classes (e.g., monoglycerides) among the three pellets.

REVIEW OF THE LITERATURE

Van Leeuwenhoek (1674) made the first recorded observations of fat globules in cows' milk.

Two comprehensive reviews of research performed on the fat globule membrane have provided information concerning the natural state of the fat globules themselves. King (1955) wrote: "Due to surface forces the fat particles [globules] occur as spheres floating in the milk plasma, . . ." Brunner (1965) stated: "Milkfat exists in the liquid state at the temperature of freshly secreted milk (39-40°C.)" and ". . . most of the fat globules found in normal unagitated cow's milk are smaller than 4 u and seldom do they exceed 10 u in diameter. Globules smaller than 1.0 u in diameter are difficult to identify and enumerate."

The discovery of the fat globule membrane was described by King (1955): ". . . Ascherson in 1840 came to the conclusion that they [fat globules in cows' milk] are enveloped in a very thin membrane."

The investigations into the size and chemical nature of the fat globule membrane were first undertaken by using light microscopy and later by using electron microscopy. A portion of the investigations, as reviewed by King (1955), showed first, the existence of the fat globule membrane, thus verifying Ascherson's "conclusion," and second, the presence of protein in the membrane. Commenting on pertinent electron microscope work, Brunner (1965) wrote: "Knoop and Wortmann estimated the membrane thickness at 5-10 mu from osmium fixed methacrylate embedded cream. Roelofsen and Salome . . . ob-

served samples of milk powder, fixed with osmium tetroxide vapor and mounted in methacrylate. They distinguished an osmiophilic layer less than 10 mu thick surrounding the fat globules."

Providing an insight into the physical state of the fat globule membrane, King (1955) wrote:

> "According to Polonovski et al. (1949), the addition of chloroform or acetone to milk provokes a swelling of the fat globules, whereby the globule diameter may increase three-fold (cf. also Hattori, 1925). The mechanism of this phenomenon is, however, obscure, and a conclusion as to distensibility of the membrane has to be taken with caution. On the other hand, the envelope around the fat globules is so 'tight' that the fat solvents are unable to extract the fat unless the membrane is chemically pretreated with alcohols, alkalis, acids, etc."

The next part of this review will examine the chemical composition of the fat globule membrane. Every researcher who has attempted to make some sort of chemical analysis of the membrane has faced the same problem, the isolation of the membrane. King (1955) summarized the problem as follows: "In order to isolate the membrane substance for investigation . . . , it has to be separated, on the one side, from the components of the aqueous phase of milk (plasma proteins, lactose, salts, etc.), and, on the other side, from the material contained inside the fat globules (the fat proper, and substances dissolved in it). The first aim is achieved by 'washing' the fat globules with water, . . ." One method of washing fat globules utilizes the centrifugal cream separator. Whole milk is separated into skim-milk and cream. The cream is diluted with water. The resulting suspension is mixed thoroughly and is then reintroduced into the separator. The process of diluting the cream with water, mixing, and reseparating is repeated as many times as the investigator

desires. This scheme was first devised and used by Storch (1897). Subsequent investigations of separator washing by Rimpila and Palmer (1935), Jack and Dahle (1937), and Brunner et al. (1953a) showed that most of the protein the milk plasma is removed by the first washing.

Völtz (1904), Abderhalden and Völtz (1909), and Titus et al. (1928) washed fat globules by allowing them to rise from whole milk through a column of water containing a preservative (as did the water itself). Recently, Sander (1962) applied the column principle in his research. He substituted a centrifuge tube for the column and separated the fat globules from the skimmilk constituents in a refrigerated centrifuge. Thus, he avoided the use of a preservative. He claimed advantages and some disadvantages for use of the centrifuge technique as compared to use of the cream separator.

The separation of the fat globule membrane from the rest of the fat globule has commonly been accomplished by churning. Palmer and Samuelsson (1924) were the first to employ this method. So universal has been the use of churning in fat globule membrane research that now the membrane is virtually defined as the material, excluding unchurned fat globules, found in buttermilk and butterserum following churning. Buttermilk, of course, is the aqueous phase released during the churning of cooled, washed cream. Butterserum is the aqueous phase released when butter is melted (the other phase is butteroil).

The amount of fat globule membrane material in cows' milk has been determined by Völtz (1904), Abderhalden and Völtz (1909), Storch (1897), Palmer and Wiese (1933), Jenness and Palmer (1945a), Schwarz and Fischer (1937), and Morton (1954).

Herald and Brunner (1957) qualitatively determined the trace elements of the fat globule membrane. They found aluminum, calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, silver, and zinc.

Extraction of the fat globule membrane with fat solvents leaves a residue which is almost entirely protein. The residue has been studied by Storch (1897), Völtz (1904), Abderhalden and Völtz (1909), Hattori (1925), Titus et al. (1928), Rimpila and Palmer (1935), Schwarz and Fischer (1937), Sandelin (1941), Jenness and Palmer (1945a), Hare et al. (1952), Brunner et al. (1953a), Brunner et al. (1953b), Herald and Brunner (1957), Brunner and Herald (1958), Brunner and Thompson (1961), and Jackson et al. (1962).

The presence of lipids in the fat globule membrane was first detected by Palmer and Samuelsson (1924). Since that time a great deal of information concerning the types and amounts of these lipids has been gained. Before examining the research done with the lipids, however, it is appropriate to present some data concerned with the total lipid fraction of whole milk.

Table 1, taken from Jenness and Patton (1959), gives the composition of the whole milk lipid fraction. Table 2 gives the percentages of phospholipid fractions from whole milk, where 100% is the entire amount of phospholipid. These tables will provide a background for discussion of the lipids of the fat globule membrane.

Palmer and Samuelsson (1924) found phospholipids associated with the fat globule membrane. Palmer and Wiese (1933) fractionated the membrane phospholipids. They identified cephalin, lecithin, and a diaminophospholipid, presumably sphingomyelin, in their fractions.

Palmer and Wiese (1933) also were the first to isolate a so-called high-melting triglyceride fraction in the fat globule membrane. This fraction has also been called the high-melting glyceride fraction. Rimpila and Palmer (1935) reported that 40.4-50.7% of the fat globule membrane consisted of ether-extractable non-phospholipid material.

Kon et al. (1944) proposed that carotenoids and cholesterol may be associated with the fat globule membrane.

An interesting finding was made by Jenness and Palmer (1945 a, b). They determined that the protein to phospholipid ratio was higher in buttermilk (2.4-3.8, mass/mass) than in butterserum (1.0-2.0, mass/mass).

The research of White et al. (1954) demonstrated that both vitamin A and the carotenoids are components of the fat globule membrane. Vitamin A was found to be 0.0483% of the membrane; the carotenoids, 0.0645%.

According to Mulder et al. (1957), 60% of the total phospholipid in whole milk is located in the fat globule membrane.

Thompson et al. (1961) separated the total lipid fraction of the fat globule membrane into general lipid classes by using a silicic acid column. Their method of isolation of the membrane lipids is shown in Figure 1. Their data is given in Tables 3 and 4.

Wolf and Dugan (1964) further characterized the high-melting triglyceride fraction. They presented data showing the amounts of saturated fatty acids and the amounts of unsaturated fatty acids located on each of the α and β positions of the triglycerides.

Morton (1954) was the first to fractionate the fat globule

membrane by differential sedimentation in the ultracentrifuge. He recovered 0.25 grams of sedimented membrane per liter of whole milk. The enzymes alkaline phosphatase, xanthine oxidase and diaphorase were found to be present in each of the three pellets which Morton isolated. He found significant amounts of lipids and some "nucleic acid phosphorous" in the pellets. Morton emphasized the similarities between his fractions and microsomes isolated from mouse tissue. Morton felt the similarities were so great that he gave the name "milk microsomes" to the sedimented fat globule membrane fractions.

Bingham et al. (1961) demonstrated the presence of acid phosphatase in the fat globule membrane.

Alexander and Lusena (1961) summarized a portion of their work as follows: "The membrane material obtained by freezing washed cream has been fractionated from suspensions in 2% sodium desoxycholate into five sedimentable fractions representing 75% of the total material and one soluble fraction (25%)." Their original membrane material contained 60.2% lipid. Twenty-nine percent of the total lipids were phospholipids. The percent phospholipids of the total lipids in each of the five pellets ranged from 5.1 to 15.0%. For the supernatant, however, the same figure was 51.0%.

Brunner (1962) also fractionated the fat globule membrane by centrifugation. By spinning the membrane at 25,000 X g for two hours he obtained a pellet and a supernatant. The pellet contained 10% lipid; the supernatant, 60%. The pellet lipid fraction contained 38.2% phospholipids. The supernatant lipid fraction contained 23.2% phospholipids.

Sander (1962) subjected the fat globule membrane to ultracen-

trifugation at 20,000 rpm for 20 hours. He reported that this treatment sedimented 82 to 87% of the membrane nitrogen. Also, he found that 47% of the sedimented pellet were lipids.

The most recent fractionation of the fat globule membrane by centrifugation was reported by Richardson and Guss (1965). Figure 2 illustrates part of their experimental procedure. A portion of their data is given in Table 5.

Table 1 Composition of the milk lipid fraction (taken from Jenness and Patton, 1959)

Component	Concentration	Component	Concentration
Triglycerides	98-99%	Fat soluble vitamins	
Phospholipids	0.2-1.0%	Vitamin A	7.0-8.5 ug/g fat
Sterols	0.2-0.4%	Carotenoids	8.0-10.0 ug/g fat
Free fatty acids	traces	Vitamin E	2.0-50.0 ug/g fat
Waxes	traces	Vitamin D	traces
Squalene	traces	Vitamin K	traces

Table 2 Relative amounts of phospholipid classes of whole milk expressed as percentages of the total phospholipid fraction of whole milk

Component	Source of data		
	Smith and Freeman (1959)	Sprecher (1963)	Morrison and Smith (1964)
	Concentration		
	(Wt %)	(M %)	(unknown)
Cerebrosides	6	no data	6
Cephalins	35	39	37
Lecithins	32	32	31
Sphingomyelins	24	26	23

Table 3 Composition of the lipid fraction of the fat-globule membrane prepared by Scheme I (see Figure 1) (taken from Thompson et al., 1961)

Lipid[a]	Peak No.	Lipid eluted from silicic acid (mg.)	Percentage of membrane lipids (%)	Percentage of whole membrane[b] (%)
Carotenoids	1	2.2	0.45	0.30
Squalene[c]	2	3.0	0.61	0.40
Cholesterol esters	3	3.9	0.79	0.54
Triglycerides	4	263.0	53.41	36.12
Free fatty acids plus other triglycerides	5	31.0	6.30	4.26
Cholesterol	6	25.4	5.17	3.50
Diglycerides	7	40.0	8.14	5.49
Monoglycerides	8	22.9	4.66	3.14
Phospholipids	9,10,11	100.0	20.35	13.76
Totals		491.4	99.88	67.51
Percentage recovery		104.3 (%)		

[a]Listed in order of elution from silicic acid column
[b]Membrane consisted of 32.49% protein
[c]Precise identification pending

Table 4 Composition of the lipid fraction of the fat-globule membrane prepared by Scheme II (see Figure 1) (taken from Thompson et al., 1961)

Lipid[a]	Peak No.	Lipid eluted from silicic acid (mg.)	Percentage of membrane lipids (%)	Percentage of whole membrane[b] (%)
Squalene	----	0	0	0
Carotenoids	----	0	0	0
Cholesterol esters	1	2.9	0.63	0.27
Triglycerides	2	230.9	49.98	21.88
Free fatty acids plus other triglycerides	3	Traces	----	----
Cholesterol	4	16.8	3.64	1.59
Diglycerides	5	48.9	10.58	4.63
Monoglycerides	6	29.8	6.45	2.82
Phospholipids	7,8,9	132.7	28.72	12.57
Totals		462.0	100.00	43.76
Percentage recovery		104.6 (%)		

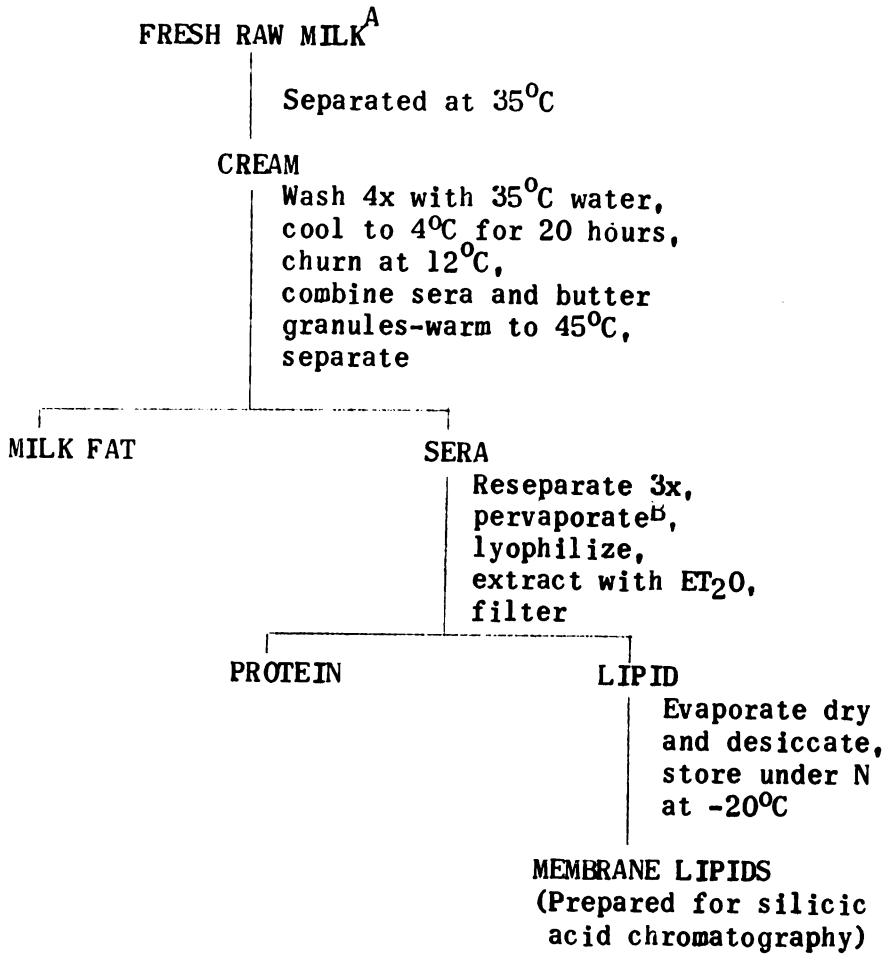
[a]Listed in order of elution from silicic acid column
[b]Membrane consisted of 56.24% protein
[c]Precise identification pending

Table 5 Gross composition of membrane fractions (taken from Richardson and Guss, 1965)

Fraction	Component				
	Crude protein[a] (%)	Lipid (%)	Phospholipid[b] (Wt % of lipid)	Nonpolar lipid[c] (Wt % of lipid)	Plasmalogen (Mole % of phospholipid)
1 (FGMM)	50.5	48.1	25.0	75.0	3.0
2 (10^4 G)	70.6	28.9	49.0	51.0	3.7
3 (M)	71.9	31.5	40.0	60.0	3.3
4 (MC)	24.3	75.4	37.5	62.5	1.3
5 (MS)	27.2	72.1	18.5	81.5	2.0

[a]Residue from lipid extract
[b]Lipid phosphorus X 25
[c]100-Phospholipid

Scheme I



Scheme II

Scheme II varies from Scheme I as follows:

A. Warm, fresh, whole milk was separated shortly after milking without being cooled.

B. The sera was washed 2x with ET_2O to remove free fat, and was not pervaporated before lyophilization.

Figure 1 The isolation of the total lipids of the fat globule membrane of cows' milk (taken from Thompson et al., 1961)

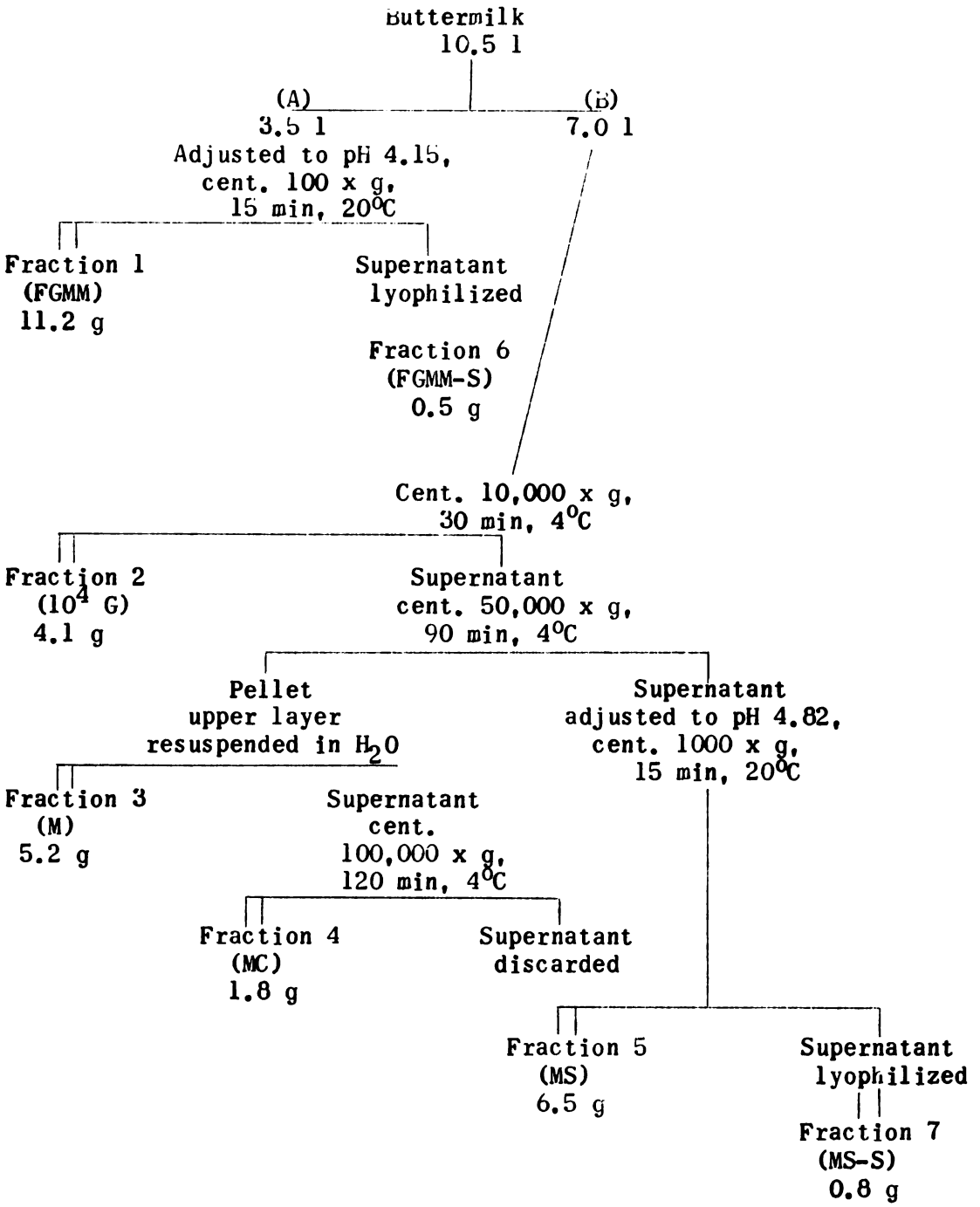


Figure 2 Fractionation of the fat globule membrane (taken from Richardson and Guss, 1965)

EXPERIMENTAL AND RESULTS

Materials

Chemicals

The following chemicals were obtained from Nutritional Biochemicals Corporation: crystalline beta carotene; animal cephalin; cholesterol; cholesterol acetate; DL alpha tocopherol (vitamin E); sodium penicillin "G" marked "Not less than 1625 units per mg."; stearic acid; and streptomycin sulphate marked "Not less than 700 micrograms per mgm."

The following chemicals were Fisher reagent chemicals: diethyl ether (anhydrous); glacial acetic acid; sulfuric acid; and thionyl chloride.

The following chemicals were Merck reagents: benzene; methyl alcohol anhydrous (methanol) marked "free from acetone"; and n-hexane.

The following chemicals were obtained from the Michigan State University Stores: ethanol, 95%; and nitrogen, in metal cylinders.

The containers of the following chemicals bore the label, Mallinckrodt Chemical Works: hydrogen peroxide solution 30% analytical reagent; and silicic acid 100 mesh (powder) $SiO_2 \cdot xH_2O$ analytical reagent.

The containers of the following chemicals bore the label, Matheson Coleman and Bell Division, The Matheson Company, Inc.: chloroform reagent A.C.S.; and ferrous sulfate reagent, A.C.S., granular.

Potassium chloride was the Fisher certified reagent.

Potassium hydroxide, U.S.P. (pellets) was the Fisher laboratory chemical.

The container of the Myverol Distilled Monoglycerides bore the label, Distillation Products Industries--Division of Eastman Kodak Co.

The bottle of ninhydrin (triketohydrindene hydrate) bore the label, Pfanstiehl Laboratories, Inc.

The bottle of potassium dihydrogen phosphate, C.P., bore the label, J.T. Baker Chemical Co.

The container of silica gel G acc. to Stahl bore the label, E. Merck AG.

Tap water was run through a Culligan exchanger. The effluent was deionized water.

Equipment

The applicator used for preparing thin layers of silica gel G on glass plates was the Research Specialties Co. Model 200.

The applicator board used for preparing thin layers of silica gel G on glass plates bore the label, Scientific Glass Apparatus Co., Inc.

Methods

Storage of Chemicals

The following chemicals were stored at -20^{o}C: beta carotene, cholesterol, cholesterol acetate, Myverol monoglycerides, and

stearic acid.

The following chemicals were stored at 35°F: alpha tocopherol, cephalin, chloroform, diethyl ether, hydrogen peroxide, methanol, and n-hexane.

The rest of the chemicals and equipment listed under MATERIALS were stored at room temperature.

Special Treatment of Some Solvents

Some solvents were redistilled in an all-glass distillation apparatus prior to use. The redistilled solvents and the boiling ranges of the redistilled solvents were the following: benzene, 80.1°C; chloroform, 58.0-61.5°C; diethyl ether, 34.6°C; ethanol, 78.5°C; n-hexane, 68.7°C; and methanol, 64.6°C.

Following redistillation of chloroform, 0.4% (v/v) methanol was added to the chloroform.

Diethyl ether was redistilled over ferrous sulfate and stored over iron nails.

Prior to redistillation of ethanol, this solvent was refluxed for two hours over pellets of potassium hydroxide.

Redistilled chloroform was stored in a glass bottle with a ground glass stopper. The five remaining redistilled solvents were stored in glass bottles with screw caps.

Redistilled benzene was stored at room temperature. The other five redistilled solvents were stored at 35°F.

Babcock Fat Test

The Babcock fat test was carried out by the method given by

the Mojonnier Bros. Co. (1925).

Explanation of Fractionation by Differential Sedimentation

The research of Morton (1954), Alexander and Lusena (1961), and Brunner (1962) has been discussed in the REVIEW OF THE LITERATURE. One conclusion which can be reached from this research is that aqueous suspensions of the fat globule membrane can be fractionated in the ultracentrifuge.

How is such a fractionation accomplished? The reader is now referred to Tanford (1961). The ensuing discussion is relevant to centrifugation of a sample in either an analytical cell or a centrifuge tube.

Tanford derived the following equation:

$$s_p = (1/r_z w^2)(dr_z/dt), \qquad (1)$$

where s_p is the sedimentation coefficient of some single type of particle i in the so-called "plateau region,"

r_z is the linear distance measured from the axis of rotation of the centrifuge to a point within the cell or tube (Tanford gives an exact definition of r_z.),

w is the angular speed of the rotor of the centrifuge, and

t is time.

Tanford also gives the definition of the sedimentation coefficient, s:

$$s \equiv M(1 - \bar{v}_2 \rho_0)/Nf, \qquad (2)$$

where M is the mass of one mole of some single type of particle i,

$\bar{v}_2$ is the partial specific volume of the particle i immersed in a solvent j,

ρ_0 is the density of the pure solvent j,

N is Avogadro's number, and

f is the frictional coefficient of particle i immersed in solvent j.

Substitute s for s_p in equation (1) and give M the subscript p:

$$(1/r_z w^2)(dr_z/dt) = M_p(1 - \bar{v}_2\rho_0)/Nf. \qquad (3)$$

Now M_p/N is just the mass of particle i, m_p. Substitution of m_p for M_p/N gives

$$(1/r_z w^2)(dr_z/dt) = m_p(1 - \bar{v}_2\rho_0)/f. \qquad (4)$$

Let r_p be the linear distance of particle i measured from and perpendicular to the axis of rotation. r_p can be used as an approximation for r_z (cf. Tanford). Substitute r_p for r_z in equation (4).

$$(1/r_p w^2)(dr_p/dt) = m_p(1 - \bar{v}_2\rho_0)/f. \qquad (5)$$

A derivative, dr_p/dt is plainly the radial velocity of particle i (i.e., it is the rate of change of r_p with respect to time). Now, $r_p w^2$ is the magnitude of the acceleration vector directed radially from the axis of rotation of the centrifuge. All objects lying in the cell or tube and at a distance r_p from the axis of rotation are subject to the acceleration $r_p w^2$.

The left side of equation (5) gives the velocity per unit acceleration of particle i. What if different particles in the same system exhibit different velocities per unit acceleration? The right side of equation (5) shows that different velocities are directly attributable to differences in the values of m_p, $\bar{v}_2$, and f among the particles. In other words, differences in the values of m_p, $\bar{v}_2$, and f among particles will result in different velocities per unit acceleration of those particles. Furthermore, because of

differences in the values of m_p, $\bar{v}_2$, and f a system of particles can be separated into fractions according to the differences in the velocities per unit acceleration.

How can such a separation be accomplished? For theoretical purposes we can start by separating the variables in equation (5):

$$dr_p/w^2 r_p = m_p(1 - \bar{v}_2 p_0)dt/f. \tag{6}$$

Perform the following definite integration:

$$\int_{r_{p1}}^{r_{p2}} dr_p/w^2 r_p = \int_0^t m_p(1 - \bar{v}_2 p_0)dt/f. \tag{7}$$

Consider that $1/w^2$ and $m_p(1 - \bar{v}_2 p_0)/f$ are constants.

$$(1/w^2) \int_{r_{p1}}^{r_{p2}} dr_p/r_p = \frac{m_p(1 - \bar{v}_2 p_0)}{f} \int_0^t dt. \tag{8}$$

$$(\ln r_p)/w^2 \Big|_{r_{p1}}^{r_{p2}} = m_p(1 - \bar{v}_2 p_0)t/f \Big|_0^t. \tag{9}$$

$$\frac{(\ln r_{p2}) - \ln r_{p1}}{w^2} = m_p(1 - \bar{v}_2 p_0)\ (t - 0)/f. \tag{10}$$

$$(1/w^2)\ln (r_{p2}/r_{p1}) = m_p(1 - \bar{v}_2 p_0)t/f. \tag{11}$$

Recall that s_p equals $m_p(1 - \bar{v}_2 p_0)/f$. Therefore,

$$(1/w^2)\ln (r_{p2}/r_{p1}) = s_p t. \tag{12}$$

The discussion can now be confined to a fixed angle rotor which holds cylindrical centrifuge tubes.

Let r_{p1} be r_n, the linear distance measured from and perpendicular to the axis of rotation to the nearest location in the interior of the centrifuge tube. Similarly, let r_{p2} be r_f, the linear distance measured from and perpendicular to the axis of rotation to the farthest location in the interior of the centrifuge tube. From the

limits of integration in equation (7) it is plain that t is the time required for particle i to move from r_n to r_f. Rewriting equation (12):

$$(1/w^2)\ln (r_f/r_n) = s_p t. \qquad (13)$$

Perhaps it is now clear that the integration of equation (6) transformed the unmanageable equation (5) into an equation, (13), in which all variables can be easily determined.

An example might be useful. Suppose that centrifuging a suspension or solution of particles results in the sedimentation of some of the particles into a pellet at the bottom of the centrifuge tube. What can be said about this pellet? By plugging the values of w, r_f, r_n, and t into equation (13), s_p could be calculated. Now s_p is the sedimentation coefficient of that type of particle i which took all of time t to migrate from r_n to the pellet at r_f. What about the faster particles? They reside in the pellet but their s_p's are higher than that of particle i since s_p is inversely proportional to t. What about particles which are slower than particle i? They are distributed in both the supernatant and the pellet. They can be removed from the pellet in the following manner. Remove the supernatant from above the pellet. Resuspend the pellet in fresh solvent. Centrifuge the suspension at the original conditions. Particles slower than particle i will again be partitioned between the supernatant and the pellet. The repeated process of removing the supernatant, adding fresh solvent to the pellet, resuspending and recentrifuging the mixture has the effect of diluting the particles slower than particle i out of the pellet. When all or nearly all of the particles slower than particle i are removed from the

pellet, then particle i is the slowest of the particles of the pellet. Since the s_p is the sedimentation coefficient of particle i, s_p has suitably been termed the "minimum sedimentation coefficient" of the pellet.

The supernatant from the preceding fractionation can be fractionated further, but one or more of the following changes in the conditions of centrifugation will have to be made: increase w; increase t; increase r_n; decrease r_f.

The foregoing discussion demonstrates the technique of differential sedimentation. This technique enabled Morton (1954), Alexander and Lusena (1961), Brunner (1962), and recently Richardson and Guss (1965) to fractionate suspensions of the fat globule membrane. Of course, reading Tanford is not requisite for performing differential sedimentation. The theory only attempts to explain why differences in velocities per unit acceleration exist. Also the sedimentation coefficient is a convenient name tag which is more or less understood.

Preparation No. 1

Collection of raw material. Cows' milk was the raw material. Cows' milk was collected at the afternoon milking of cows of the Michigan State University Holstein Herd. Cows whose milk was collected were selected randomly on the day of collection. Milk was collected from the cow's udder in a Surge bucket. When the udder was empty the milk was immediately transferred from the bucket to a ten-gallon milk can. When ten gallons were obtained, collection ceased. The collected milk was then transported to the location of

a cream separator.

Isolation of an aqueous suspension of the fat globule membrane of cows' milk. A laboratory-size De Laval Model 9 centrifugal cream separator was used to accomplish the separation of milk into skimmilk and cream. The separator was turned on and allowed to develop its maximum speed. Tap water at 40°C was run through the separator for a brief period. Immediately following the warming of the separator with the tap water, the milk was intorduced into the separator. The skimmilk was discarded.

Following separation the separator was turned off, disassembled, cleaned, reassembled, turned on, and again allowed to reach its maximum speed. The separated cream was diluted to three times its original volume with 40°C tap water. The cream-and-water suspension was mixed thoroughly. The separator was prewarmed with tap water as before. The cream suspension was introduced into the separator. The aqueous stream from the skimmilk spout of the separator was discarded. The cream was kept.

The foregoing process of diluting separated cream with water, mixing, and reseparating is one washing operation. A total of five washing operations were performed. In the first two the cream was diluted to three times its original volume. In the last three washing operations the cream was diluted to four times its original volume.

The separator itself was not disassembled following any of the several washings. After each washing cold water first, then the hottest tap water was run through the running separator. Excellent cleaning of the cream side of the separator was accomplished by

running a high velocity stream of the hottest tap water directly into the separator's cream spout.

The foregoing depicts how washed cream was obtained. The next step in the isolation of the fat globule membrane was churning.

For the purpose of churning it was considered desirable that the fat content of the washed cream be 35 weight per cent. The fat content of one lot of washed cream was determined to be 52% by the Babcock fat test. A different lot of washed cream tested 52.53% fat by the Mojonnier fat test. This latter value was used in subsequent calculations. An extrapolation of the data of Jenness and Patton (1959) gave 0.965 g/ml as the density of cream containing around 52.53% fat.

The adjustment of the per cent fat of the washed cream from 52.53% to the desired 35% was accomplished by adding tap water to the cream. The density of the tap water was assumed to be 1 g/ml. The calculation of the exact volume of tap water to add to the cream was made in the following manner.

Let V be the volume of washed cream.

Let X be the volume of tap water to be added to the washed cream.

Since the cream will contain the same mass of fat both before and after the addition of tap water, an equation can be written:

$$\frac{52.53 \text{ g fat}}{100 \text{ g}} \left(0.965 \frac{\text{g}}{\text{ml cream}}\right) V = \frac{35 \text{ g fat}}{100 \text{ g}} \left\{0.965 \frac{\text{g}}{\text{ml cream}} (V) + \frac{1 \text{ g}}{\text{ml } H_2O} (X)\right\}. \quad (14)$$

$$\frac{52.53(0.965)(V)}{35} \frac{\text{g}}{\text{ml cream}} = 0.965 \frac{\text{g}}{\text{ml cream}} (V) + \frac{1 \text{ g}}{\text{ml } H_2O} (X) \quad . (15)$$

$$X = 0.965\ (V)\ \frac{g}{ml\ cream}\left(\frac{52.53}{35} - 1\right)\frac{ml\ H_2O}{g} . \quad (16)$$

$$X = 0.965\ (V)\left(\frac{52.53-35}{35}\right)\frac{ml\ H_2O}{ml\ cream} . \quad (17)$$

$$X = 0.965\ (V)\left(\frac{17.53}{35}\right)\frac{ml\ H_2O}{ml\ cream} . \quad (18)$$

$$X = 0.483\ (V)\ ml\ H_2O/ml\ cream . \quad (19)$$

X was added to the washed cream. The cream-and-water suspension was mixed thoroughly. The suspension was then poured into wide-mouth screw-top glass jars. The volume of suspension in any jar was limited to less than one-half of the interior volume of the jar. The temperature of the suspension in the jars was adjusted to 8°C. The headspaces of the jars were flushed with nitrogen. Jar caps were screwed onto the jars. One jar was placed into a mechanical agitator. During agitation the motion of the jar in the agitator resembled that of a piston in a reciprocating gasoline engine.

The agitation churned the washed cream, i.e., the cream suspension was transformed into butter and buttermilk. Agitation of the cream suspension continued until no small butter particles were visible.

The remaining suspension in the other glass jars was similarly churned.

Each churned mass (butter plus buttermilk) was warmed until the butter melted completely to liquid. It was never necessary to exceed 50°C while melting the butter. The liquid mass was then transferred to a large separatory funnel. The headspace of the funnel was flushed with nitrogen. The funnel was stoppered and shaken. When a sharply defined interface between the lower aqueous

phase and the upper butter oil was visible, the aqueous phase was carefully drawn from the funnel. The volume of the aqueous phase was measured in a graduated cylinder and recorded. The aqueous phase, a suspension of the fat globule membrane plus some unchurned fat globules, was stored under nitrogen at $2^{o}C$.

Fractionation of an aqueous suspension of the fat globule membrane by differential sedimentation in the ultracentrifuge. The sedimentation coefficient s_p which appears in equation (13) has units of time. For convenience the Svedberg unit has been defined as follows:

$$10^{-13} \text{ seconds} = 1 \text{ Svedberg unit} \qquad (20)$$

The abbreviation of Svedberg unit is S.

The original experimental design of this research called for the separation of an aqueous suspension of the fat globule membrane into fractions by ultracentrifugation. By using the technique of differential sedimentation three pellets would be collected. The minimum sedimentation coefficients of the three pellets would be 400S, 80S, and 40S. Each of these pellets would be analyzed for the major lipid classes: hydrocarbons, cholesterol esters, triglycerides, fatty acids, cholesterol, diglycerides, monoglycerides, cephalins, lecithins, and sphingomyelins.

All ultracentrifugation was done in a Beckman/Spinco Model L Preparative Ultracentrifuge. The Type 21 Rotor, a fixed angle rotor, was always used. The Type 21 Rotor holds ten centrifuge tubes. The polypropylene centrifuge tube, with a capacity of about 83 ml, was the type used.

The reader is now referred to equation (13). The Spinco

Instruction Manual LIM-2 (Spinco Division, 1962) provides values for r_n, 6.0 cm, and r_f, 12.0 cm. Also it gives the maximum speed of the Type 21 Rotor as 21,000 rpm. From 21,000 rpm a w can be calculated. By plugging the values of r_n, r_f, and w into equation (13), $s_p t$ had been calculated as 430 (S)(hours).

The time required to sediment the 400S, 80S, or 40S pellet was calculated from the equation

$$t = 430 \text{ (S)(hours)}/s_p . \qquad (21)$$

Table 6 gives the calculated time required to sediment a pellet.

The pellets were not washed. The original membrane suspension was centrifuged at 21,000 rpm for 64.5 minutes. The pellet and an aliquot of the supernatant were stored. The remaining supernatant was then centrifuged at 21,000 rpm for 323 minutes. Again, the pellet and an aliquot of the supernatant were stored. The remaining supernatant was centrifuged at 21,000 rpm for 645 minutes. The pellet and final supernatant were stored. Storage for the fractions mentioned was at $2^{\circ}C$ under nitrogen.

Mojonnier fat tests and total solids tests were made on the original membrane suspension and each of the supernatants. The results are given in Table 7.

The data in Table 7 reveals that the membrane suspension contained much more solids and lipids than did the 400S supernatant. The differences in the percentages of total solids and lipids between the 400S supernatant and the 80 and 40S supernatants were not nearly as large. The conclusion was that the amounts (masses) of the 80 and 40S pellets were much smaller than that of the 400S pellet. The amounts of 80 and 40S pellets were so small, in fact, that it was

considered that there would not be enough material for analysis, assuming that about 830 ml of membrane suspension was the starting material for ultracentrifugation.

Visual inspection of the three pellets verified the above conclusion. Further, it was seen that the 80S and 40S pellets were very similar both in amount and appearance.

In short, the ultracentrifugation portion of the original experimental design was considered unsatisfactory.

A new scheme for centrifugation was drawn up. Again, a membrane suspension would be fractionated into three pellets and a final supernatant by differential sedimentation in the ultracentrifuge. The minimum sedimentation coefficients of the three pellets would be chosen so that the amount of each pellet would be sufficient for analysis. Finally, the fractionation would be a bit more meaningful if significant differences in the appearances of the pellets were observed.

It was decided that the fractionation would roughly follow the scheme used in the fractionation of cell constituents by centrifugation. That is, the minimum sedimentation coefficients of the pellets would correspond to those of (1) cellular debris, (2) mitochondria, (3) microsomes, (4) polysomes, and (5) ribosomes.

The conditions for sedimenting cellular debris were found to be 2,500 for 10 minutes. The first application of these conditions to a membrane suspension demonstrated that it was not feasible to collect a pellet corresponding to "cellular debris." The pellet was so loosely packed that its redispersion was initiated in the processes of removing the rotor from the centrifuge and the tubes from the

rotor. This fraction, therefore, was eliminated from the experimental design.

Allfrey (1959) stated that centrifugation at 5000 x g for 10 minutes would sediment mitochondria. In the application of these conditions to the membrane suspension the time was lengthened from 10 minutes to 20 minutes to insure that the pellet would be packed solidly. Further, it was necessary to convert 5000 x g to a number of revolutions per minute. This was done in the following manner.

$$\text{Centrifugal force} = mw^2r. \tag{22}$$

It is apparent that centrifugal force is dependent on the choice of a value of r. r_n was chosen. When force is given in units of gravities, the mass under acceleration is understood to be 1 gram. The gravity is defined as 980.665 dynes.

$$5000 \times g = (1 \text{ gram})(w^2)(6.0 \text{ cm}) \frac{1 \text{ gravity}}{980.665 \text{ dynes}} . \tag{23}$$

$$w^2 = (5000)(980.665)/6.0 \text{ sec}^2 . \tag{24}$$

$$w = 910/\text{sec} . \tag{25}$$

In rpm

$$w = (910/\text{sec})(1 \text{ rev}/2\pi)(60 \text{ sec/min}) . \tag{26}$$

$$w = 8{,}600 \text{ rpm} . \tag{27}$$

The speedometer on the centrifuge could be read to about ±500 rpm. Therefore w was rounded off to 8,500 rpm.

s_p could now be calculated for this pellet with the aid of equation (13). Solving for s_p gives

$$s_p = (1/w^2t)\ln (r_f/r_n) . \tag{28}$$

$$s_p = \frac{(10^{13}\text{S/sec})\ln (12.0/6.0)}{\left\{(8{,}500 \text{ rpm})\left[\frac{2\pi}{\text{rev}}\right]\left[\frac{1 \text{ min}}{60 \text{ sec}}\right]\right\}^2 (20 \text{ min})\left[\frac{60 \text{ sec}}{\text{min}}\right]} . \tag{29}$$

$$s_p = 7{,}500S . \tag{30}$$

Allfrey (1959) stated that centrifugation at 54,000 x g for one hour would sediment the microsome fraction. In the application of these conditions to the fractionation of the membrane suspension it was again necessary to find the rpm from the given centrifugal force. This was accomplished in an analogous manner to that shown in equations (22) through (27). The result was

$$w = 29{,}000 \text{ rpm} \tag{31}$$

By use of equation (28) s_p was found to be 230S. 29,000 rpm was above the maximum speed of the Type 21 Rotor. Therefore, the length of centrifugation had to be calculated based on a rotor speed of 21,000 rpm.

The derivation of equation (21) has already been described. When the derivation was actually performed, however, 430 (S)(hours) was found to be an erroneous value of $s_p t$. A calculation showed the real value to be 410 (S)(hours). Thus, the length of centrifugation of the 230S pellet was calculated from the equation

$$t = 410 \text{ (S)(hours)}/s_p \tag{32}$$

and was found to be 110 minutes.

Morris (1964) gave the sedimentation coefficient of polysomes as about 170S. The first time the 170S pellet was collected from a membrane suspension two things were determined. First, the amount of this pellet was very small. Second, collecting this fraction only meant that less material was available for the final pellet, that corresponding to ribosomes. Therefore, it was decided that the 170S pellet would not be collected.

35S was chosen as the sedimentation coefficient of the pellet corresponding to a pellet of ribosomes. The length of centrifugation

at 21,000 rpm was calculated using equation (32). For the 35S pellet t was equal to 720 minutes.

Table 8 summarizes the details of the fractionation of the membrane suspension by differential sedimentation in the ultracentrifuge. The temperature of the system being centrifuged was about 0°C.

Theoretically, the 230S pellet would contain no particles with s_p's equal to or greater than 7,500S. Similarly, the 35S pellet would contain no particles with s_p's equal to or greater than 230S. These ideas follow directly from the explanation of differential sedimentation given above. The supernatant from a pellet with a certain s_p can be fractionated (providing the supernatant contains sedimentable particles) into a pellet and supernatant by adjusting the conditions of centrifugation.

Once ultracentrifugation of a membrane suspension was underway, a serious problem arose. It appeared that microorganisms were growing in the fractions while the fractionation was in progress. The microorganisms would first appear as a white mass at the bottom of the pellet in a centrifuge tube. The first appearance would come about four days from the time the milk was drawn from the cows. It was observed that the size of the white mass would grow slightly with each redispersion and recentrifugation of the pellet.

One redispersed pellet which contained the "white mass" was smeared onto a microscope slide and stained with crystal violet. Under the light microscope many short rods were observed.

The fractions were always centrifuged and stored at temperatures which ranged from about 0 to 3°C. It was apparent that these

temperatures did not stop the growth of the short rods. The short rodes were probably psychrophiles.

It was decided to use antibiotics to try to stop the growth of the bacteria. Penicillin "G" was added to the membrane suspension at the rate of 4.6 mg/liter of suspension. Streptomycin sulfate was also added, it at the rate of 75 mg/liter. A large volume of deionized water was needed during the fractionation for redispersing the pellets. A known volume of this water was placed in a large screw cap polyethylene tank. Both penicillin "G" and streptomycin sulfate were added to this water. The final concentrations of the two antibioties in the deionized water were the same as given for the membrane suspension. The antibiotic-containing water was stored at $2^{o}C$.

Immediately following ultracentrifugation of a membrane suspension, it was observed that a cream-like layer resided at the innermost (shortest distance from the axis of rotation) surface of the suspension in the centrifuge tube. This layer was skimmed off and treated exactly as washed cream was treated prior to churning. When churned, the treated layer was transformed into a butter-like mass and an aqueous phase. The butter-like material was not yellow in color; rather it was a dull white.

It seemed that the cream-like layer was composed of unchurned fat globules. The mass of the fat globule membrane is only about 1% of the mass of the fat globule. Therefore, it was decided that the cream-like layer would be discarded during the fractionation.

The volume of the membrane suspension of preparation No. 1 was 1240 ml. Of this volume 940 ml were subjected to fractionation.

The s_p of the first pellet collected was 7,500S. Now, a

pertinent question arises. How was it known that particles with s_p's smaller than 7,500S were removed from the 7,500S pellet? The membrane suspension was cloudy. The antibiotic solution used for redispersing the pellet was clear. When, following a redispersing of the pellet and recentrifuging, the supernatant above the pellet appeared relatively clear, this was considered an indication that the supernatant was particle-free. And if the supernatant was particle-free, it meant that the pellet was free of particles whose s_p's were smaller than 7,500S.

The clarity of the supernatants above the 230S and 35S pellets was used as a criterion for the absence from those pellets of particles whose s_p's were smaller than 230S and 35S, respectively.

Six redispersions and recentrifugations were required to produce the 7,500S pellet. Eleven were required for each of the 230S pellet and the 35S pellet.

It appeared that the antibiotics succeeded in stopping the growth of bacteria in the fractions. No white masses appeared in any pellet through eleven days of centrifugation required to obtain the three pellets.

When the supernatant above a pellet was considered sufficiently clear, the pellet was recovered and redispersed in the antibiotic solution. The three pellet suspensions were stored under nitrogen at $2^{o}C$.

Appearances of the three pellets and determination of the total solids of each pellet. The 7,500S pellet was snow white in color and homogeneous in appearance. The 230S pellet consisted of two distinct parts. At the base of the pellet the material was

tea-brown in color and very solidly packed. Above this portion the material was more loosely packed and resembled the 7,500S pellet. The 35S pellet resembled the brown portion of the 230S pellet. It was homogeneous in appearance and had a hard-jelly-like texture. It was also translucent to visible light. The final supernatant had a brownish cast and resembled apple cider in appearance.

Several analyses performed on each of the three pellet suspensions will now be described. No analyses were performed on the final supernatant.

The total mass of a pellet suspension was obtained in the following manner. An Erlenmeyer flask of appropriate size was tared on a Mettler balance. The pellet suspension was added to the flask. The flask and its contents were weighed on the balance. The total mass of the suspension was the difference between the two weighings.

Total volume was measured by pouring the pellet suspension into a one liter graduated cylinder and reading off the volume.

The densities of the pellet suspensions were obtained by use of a pycnometer. The pycnometer was tared at 22.0°C on an analytical balance. The pycnometer was then filled with deionized water and the filled vessel was again weighed at 22.0°C. With the assumption that the water was pure, its density was found in the Handbook of Chemistry and Physics (1962). The pycnometer was then filled with the pellet suspension and the vessel and its contents were weighed. The density of the pellet suspension was found by the following equation:

$$d = p(s - m)/(w - m), \tag{33}$$

where d is the density of the pellet suspension,

p is the density of pure water at 22.0°C,

s is the mass of the pycnometer filled with pellet suspension,

m is the mass of the pycnometer at 22.0°C, and

w is the mass of the pycnometer filled with deionized water at 22.0°C.

A Mojonnier total solids test was made on each of the three pellet suspensions. The results of the total solids tests are given in Table 9. With the data from these tests the total solids of each pellet could be calculated in the following manner.

Let m be the mass of the total solids of a pellet.

Let b be the mass of the total solids of the pellet actually isolated.

Since only 940 ml of the 1240 ml of membrane suspension was actually fractionated, an equation can be written:

$$m = 1240b/940. \tag{34}$$

Now, if M is the mass of the isolated membrane suspension and f is the fraction of the suspension which is solids, then

$$b = Mf \tag{35}$$

f is given by

$$f = (1/n)\sum_{i=1}^{n} \frac{t_i - (cT_i/d)}{T_i}, \tag{36}$$

where n is the number of individual total solids tests included in the calculation,

t_i is the mass of total solids in the ith test,

T_i is the mass of the pellet suspension used in the ith test,

d is the density of the pellet suspension, and

c is the mass of antibiotics per unit volume of antibiotic solution.

With the exception of "particle i" the symbol i represents the code number of an individual determination. Multiple determinations are coded by successive integers beginning with 1 for the first determination.

In deriving equation (36) the assumption was made that c was the same as the concentration of antibiotics in the pellet suspension.

Equation (36) can be simplified.

$$f = (1/n)\sum_{i=1}^{n}\left[(t_i/T_i) - (c/d)\right] .$$

$$f = (1/n)\left[\sum_{i=1}^{n}(t_i/T_i)\right] - (1/n)\sum_{i=1}^{n}(c/d) . \qquad (37)$$

$100t_i/T_i$ is the per cent total solids as determined by the Mojonnier total solids test.

Let

$$(1/n)\sum_{i=1}^{n}(t_i/T_i) = a . \qquad (38)$$

100a is the mean value of the per cent total solids as determined by the Mojonnier total solids test.

Equation (37) can be further simplified.

$$f = a - (1/n)(nc/d) .$$

$$f = a - (c/d) . \qquad (39)$$

A combination of equations (34), (35), and (39) gives

$$m = 1240\ M\left[a - (c/d)\right]/940 . \qquad (40)$$

The value of c was $(7.96/10^5)$(grams/ml) .

Pellet suspension masses, pellet suspension densities, total solids data, and appearances of the 7,500S, 230S, and 35S pellets are given in Table 10.

General scheme for the quantitative determination of the major lipid classes of the three pellets. The quantitative determination of the major lipid classes in the pellets was carried out according to the following general scheme. First, the total lipids were extracted from the pellet suspension. The total lipids were then separated into the neutral lipids and the polar lipids. The major lipid classes in each of these two groups were then determined by quantitative thin-layer chromatography.

Extraction of total lipids from the pellet suspensions. Our objective in extracting the lipids from the pellet suspensions was this: to extract the major lipid classes in their entirety and to extract nothing else.

The first method of lipid extraction considered was that used in the Mojonnier fat test. This method was of doubtful value, however, since Haven and Levy (1941) showed that mixtures of chloroform and methanol were more effective in the extraction of sphingolipids from tissue than were mixtures of diethyl ether and ethanol. Ways and Hanahan (1964) showed that a procedure utilizing chloroform and methanol as extracting solvents extracted a greater amount of phospholipids from human red cells than did a precedure utilizing diethyl ether and ethanol. Duthrie and Patton (1965) showed that two simple modifications of the extraction procedure of the Mojonnier fat test increased the amount of phospholipids extracted from whole milk. First, they omitted ammonium hydroxide from the procedure. Second, they added 150 milligrams of sodium chloride to the 10 grams of milk to be extracted. They wrote: "From the structure of P-lipids known to exist in milk, it is apparent that a number of them have acidic

groups and would exist as ammonium salts in the basic aqueous phase (particularly phosphatidyl serine and phosphatidic acids), thus making them difficult to extract."

Recently, Galanos and Kapoulas (1965) cautioned against the use of ethanol as a solvent for the extraction of lipids. They recommended the use of mixtures of chloroform and methanol.

Thus it seemed that a mixture of chloroform and methanol would be the best extraction solvent. The idea for the extraction procedure finally adopted was taken from Ways and Hanahan (1964). The procedure follows:

A disc of Whatman No. 2 paper was folded into a semi-fluted form and placed into a glass funnel. The paper was washed, first with methanol, then with chloroform. Beneath the funnel was positioned a clean 500 ml glass separatory funnel equipped with a Teflon stopcock.

A Teflon covered magnetic stirring bar was placed inside a 600 ml glass beaker. One hundred and twenty-five ml of methanol were added to the beaker. The beaker was placed on a magnetic stirrer, and the methanol was stirred. Approximately 25 ml of accurately weighed pellet suspension were added to the beaker. The mixture was slowly stirred for 30 minutes at room temperature. At the end of the 30 minutes 125 ml of chloroform were added to the beaker. Slow stirring continued for 10 minutes.

At the end of the 10 minutes the contents of the beaker were poured into the Whatman No. 2 paper. Care was taken to prevent the stirring bar from dropping onto the paper. When the last of the extraction mixture had been poured from the beaker into the filter, a small aliquot of chloroform was added to the beaker. The chloroform was swirled in the beaker and poured into the filter. Adding chloroform to the beaker, swirling, and pouring into the filter was repeated several times. The total volume of these aliquots of chloroform was 125 ml. The funnel and paper were removed from the top of the separatory funnel. Fifty ml of 0.015 N aqueous potassium chloride were added to the separatory funnel. The separatory funnel was swirled briefly. The headspace above the extraction mixture was flushed with nitrogen and the separatory funnel was stoppered. The separatory funnel and its contents were vigorously shaken. The shaking was occasionally interrupted to allow excess pressure to escape through the stopcock. When shaking was finished, the stopper was removed, the headspace was again flushed with nitrogen, and the funnel was restoppered. The funnel and its contents were stored overnight at 35^{o}F. During the first hour of refrigerated storage of the funnel it was always necessary to tighten the Teflon stopcock to to prevent leakage from the funnel. This action was necessary presumably due to the fact that the coefficient of thermal expansion of Teflon is larger than that of glass.

The following morning two clear phases and a clear interface were observed in the funnel. The lower phase was drawn off and stored under nitrogen in a glass stoppered round bottom flask at -20^{o}C.

It was, of course, of interest to compare the amount of lipid

extracted from a pellet suspension by the procedure just outlined with the amounts extracted by other procedures. Four procedures were compared. The first was the Mojonnier fat test. The second followed the Mojonnier fat test except that no ammonium hydroxide was used. The third procedure followed the Mojonnier fat test except that 1.5 ml of aqueous 0.67 N potassium chloride was substituted for the 1.5 ml ammonium hydroxide usually employed. The fourth procedure began with the chloroform-methanol extraction technique outlined above. It continued by the placing of the round bottom flask onto a Rinco rotary evaporator. The bulb of the flask rested in a 40°C water bath. A vacuum within the flask was created with the aid of a water aspirator. Evaporation continued until the solvents were removed from the flask. The flask was removed from the evaporation apparatus. A small aliquot of benzene was added to the flask, swirled and poured into a Mojonnier fat test dish. The adding of benzene, swirling, and pouring into the dish was repeated several times. The dish and its contents were then treated by the methods of the Mojonnier fat test. The results of these analyses are given in Table 11. It is appropriate, perhaps, to say that these results were obtained one month prior to the publication or the author's cognizance of the research of Duthrie and Patton (1965).

Because of the results in Table 11 and because of the widespread acceptance of chloroform and methanol mixtures as lipid extracting solvents, the chloroform-methanol procedure was adopted for extraction of the lipids of the pellet suspensions of preparation No. 1.

Determination of the amount of lipid in each pellet. The

determination of the amount of lipids in each of the pellets was made in the following manner.

The round bottom flask containing the extracted lipids was removed from the -20°C storage and placed onto a Rinco rotary evaporator. The bulb of the flask rested in a 40°C water bath. A vacuum within the flask was created with the aid of a water aspirator. When the solvents had evaporated, the flask was removed from the evaporation apparatus. A small aliquot of benzene was added to the flask. The flask was swirled and the contents were then poured into a small, tared, glass beaker. The adding of benzene to the flask, swirling, and transferring to the beaker was repeated several times. The beaker was then placed on a hot plate set at 40°C. A stream of nitrogen was directed on the contents of the beaker until the benzene was evaporated. The beaker and its contents were placed inside a vacuum oven set at 40°C. A vacuum greater than 29 inches mercury was created inside the oven for one hour. The beaker was removed from the oven and placed into the cooling chamber of the Mojonnier Milk Tester. Cooling proceeded for seven minutes after which time the beaker and its contents were weighed on an analytical balance.

The amount of lipid in the entire pellet can be expressed by the following equation:

$$L = (1240M/940)(1/n)\sum_{i=1}^{n}(h_i/H_i), \qquad (41)$$

where L is the mass of lipid in a pellet,

1240M/940 is the same as in equation (40),

n is the number of individual determinations of the

amount of lipid in a pellet suspension,

h_i is the mass of lipid found in the ith determination, and

H_i is the mass of pellet suspension used in the ith extraction.

Table 12 gives the values of $100h_i/H_i$, $(100/n)\sum_{i=1}^{n}(h_i/H_i)$, and L found for the three pellets.

The amount of the pellet total solids which is lipid can now be calculated. The results are given in Table 13.

Separation of total lipids into neutral and polar lipids. Determination of relative amounts of neutral and polar lipids of each pellet. Immediately following the weighing of the extracted lipids on the analytical balance, the lipids were dissolved in chloroform. The solution of lipids was applied directly from the beaker to a column of silicic acid. The neutral lipids were eluted from the column with chloroform, the polar lipids with methanol-water, 97:3 (v/v). The complete details of preparing and using the silicic acid column are given by Ways and Hanahan (1964).

The two fractions of eluate from the column were stored at -20°C under nitrogen in round bottom flasks with ground glass stoppers. Prior to applying the lipids of one of the fractions to a thin layer of silica gel G, the solvents were removed in vacuo. The lipids were transferred to two small, tared, glass beakers. Excess solvent was removed with a stream of notrogen. The lipids were then dried in vacuo at 40°C for one hour and weighed. This procedure provided the results given in Table 14.

Thin layer chromatography. Preparation of thin layers of

silica gel G. Qualitative and quantitative determination of the major classes of neutral lipids of the three pellets. The experimental design of this research called for the separation and quantitative determination of the major lipid classes in each of the pellets. It was decided that thin-layer chromatography (TLC) would be the primary technique in this work.

Square glass plates, 20 cm square, were washed with detergent and brush. They were rinsed thoroughly with deionized water and allowed to dry. After the rinsing the plates were kept covered to prevent dust from falling on them.

Thirty grams of silica gel G were weighed into a ground glass stoppered Erlenmeyer flask.

When dry, the plates were placed on the applicator board. The metal applicator was put into place.

Sixty ml of deionized water were added to the silica gel G. The flask was stoppered and the mixture was thoroughly shaken for about 30 seconds. The mixture was poured into the applicator. The applicator was pulled across the plates. This action produced a uniform, thin layer of silica gel G on the glass plates. The adsorbent was allowed to dry. Scraping of silica gel G from and adjacent to two opposite edges of each plate produced two very sharp boundaries of the adsorbent on the plate. The scraped areas extended the length of the plate and were parallel to the direction of development which was to come later. Scraping aided in producing a straight solvent front during development. The solvent migration on unscraped plates was observed to lag behind at the edges and forge ahead in the middle, thus producing a curved front.

For chromatographing the neutral lipids, a solvent system of hexane-diethyl ether-acetic acid, 79:21:1 (v/v/v), was found to be most satisfactory. The coated plates were predeveloped with this solvent prior to the application of the neutral lipids to the plates. All developing took place in a closed glass tank. It was hoped that predevelopment, carried out in the same direction as development, would move impurities in the silica gel G to the top of the plate.

During predevelopment the migration rate of the solvent front on each plate was noted and recorded. If the migration rates of the solvent fronts of two plates were nearly identical, these two plates were chosen for subsequent work. After predevelopment the two plates were allowed to dry. Then they were activated in a hot air oven at 110°C.

It was mentioned above that the lipids of each of the two fractions isolated from the silicic acid column were divided between two tared beakers and weighed. Following weighing of the neutral lipid-containing beakers, benzene was added to both beakers. The amount of benzene added did not exceed the minimum amount required to dissolve the lipids.

After one hour of activation of the two plates, they were removed from the hot air oven and allowed to cool briefly in a desiccator. When cool, one of the plates was removed from the desiccator. The solution of lipids in benzene was applied in a band across the entire width of the plate. The band was located 2.5 cm from the bottom of the plate. The band consisted of overlapping circular spots of lipid. The spots of lipid solution were applied to the plate with a small melting point tube with two open ends.

When the entire band had been applied, the plate was placed back in the desiccator. The second plate was then taken out. The lipid solution from the second beaker was applied to the second plate in the same manner as described for the first plate. The two plates were then developed simultaneously.

During development the residual lipids on and in the two melting point tubes were washed with benzene back into the beakers from where they came. The two beakers were covered and stored.

When the solvent fronts were two cm from the tops of the plates, the plates were removed from the glass tank. One plate was placed into another glass tank. The interior of this tank was flushed with nitrogen and covered tightly with Saran Wrap. This tank was stored for the moment at -20°C.

The second plate was sprayed with concentrated sulfuric acid from an atomizer. Following the spraying the plate was heated at 110°C for one hour in a hot air oven. After one hour the plate was removed from the oven. The reaction of concentrated sulfuric acid with lipids produces carbon. The carbon is black and is readily visible when present in sufficient quantity on the TLC plate.

Figure 3 is a photograph of a thin layer chromatogram which was treated exactly as described above. The black carbon shows the positions of the neutral lipids after development.

Identification of the bands in Figure 3 was aided by some preliminary qualitative TLC. Figure 4 is a photograph of one of the preliminary plates. The plate was prepared and developed as described above. Eight reference compounds and the total lipids of the 7,500S pellet were applied separately, one compound to a spot, to the plate.

The Rf of one spot of the pellet lipids is about the same as that of stearic acid. This spot was considered to be free fatty acids and corresponds to the band in the middle of the plate of Figure 3. Another spot of the pellet lipids seems to match cholesterol. When a plate of neutral lipids is sprayed with concentrated sulfuric acid, cholesterol and cholesteryl esters develop color much faster than the other types of lipids. The spot that matches cholesterol developed color as fast as cholesterol. Therefore, it was considered to be cholesterol. This spot corresponds to the band in Figure 3 which has an Rf of about 0.3 and which follows the band of fatty acids.

Another spot of the 7,500S pellet lipids lines up with the darkest spot of the hydrogenated tallow. Triglycerides are the major constituent of tallow, and so the darkest spot should correspond to triglycerides. The spot of the pellet lipids was therefore considered to be triglycerides. This spot corresponds to the band in Figure 3 which has an Rf of about 0.9 and which lies ahead of the band of fatty acids.

In Figure 4 the major portion of the monoglyceride stayed at the origin. Therefore, the band at the origin in Figure 3 was thought to be monoglycerides.

Since it is the total lipids of the 7,500S pellet which are seen in Figure 4, the streak near the origin of the pellet lipid row was thought to be phospholipids. Partly covered by the streak and near its tip lies a single spot. This spot lines up exactly with spots seen in the hydrogenated tallow row and in the monoglyceride row. The spot in the monoglyceride row is almost invisible due to

its lack of quantity and contrast on the thin layer chromatogram. Since the monoglycerides are known to contain some diglycerides, it is a reasonable assumption that the three spots in question are diglycerides. Again, due to a lack of quantity and contrast the diglycerides are not visible in Figure 3. However, they were visible on the thin layer chromatogram. The band of diglycerides was located between the bands of monoglycerides and cholesterol.

The final band in Figure 3 to be considered lies at the solvent front just ahead of the triglyceride band. Thompson et al. (1961) and Brunner (1962) reported the presence of carotenoids, squalene, and cholesterol esters in the lipids of the fat globule membrane. It was considered that the band at the solvent front, the leading band, could contain all these lipids. But it was noticed in Figure 4 that cholesterol acetate ran behind the solvent front. If the cholesteryl esters of the long chain fatty acids migrated at the same rate as cholesterol acetate, then the leading band shown in Figure 3 would not contain cholesteryl esters. Since no long chain cholesteryl esters were immediately available, it was decided to synthesize some cholesteryl stearate.

Small amounts of cholesterol and stearic acid were dried in vacuo at room temperature overnight. The stearic acid was added to an excess of thionyl chloride in a small round bottom flask. The mixture was warmed below the boiling point of thionyl chloride, $77^{o}C$, for about one hour. Then a small amount of cholesterol was added. The mixture was heated to boil off the thionyl chloride. A thin layer plate was prepared as described above. The mixture was dissolved in benzene and applied as a band which extended one-third

of the way across the plate. Another one-third of the origin was a band of 7,500S pellet total lipids. The final one-third was a band of 7,500S pellet neutral lipids. The plate was developed in hexane-diethyl ether-acetic acid, 73:25:2 (v/v/v). The results can be seen in Figure 5. The reaction mixture lies on the left one-third of the plate. The dark streak at the solvent front developed its color as rapidly as the darker cholesterol band below when the plate was sprayed with concentrated sulfuric acid. The remaining solvent front did not exhibit fast color development. Fast color development of the solvent front band was never observed with the neutral lipids of any of the three pellets.

Thus, it seemed that the dark streak at the solvent front in Figure 5 was cholesteryl stearate. Therefore, although long chain cholesteryl esters could be in the solvent front band of Figure 3, the determination of their actual presence there will require further work.

Thus, the bands in Figure 3 are quasi-identified from the origin to the solvent front as monoglycerides, diglycerides (not visible), cholesterol, fatty acids, triglycerides and the unknown leading band. TLC of the neutral lipids of all three pellets produced the same pattern seen in Figure 3.

The proposed identifications of the neutral lipids are strongly supported by the work of Wood *et al.* (1964), Schlierf and Wood (1965), Privett *et al.* (1965), and Mangold (1961).

The idea for the quantitative technique was taken from Komarek *et al.* (1964).

When one plate had been sprayed and the black bands were

clearly visible, the second plate was removed from its temporary cold storage. The two plates were placed side by side. The unsprayed plate was marked with a wax pencil according to the locations of the bands on the sprayed plate.

A rubber stopper was fitted to the stem of an ultra-fine fritted glass filter, two cm in diameter. The stopper fit the mouth of special test tubes which were fitted with sidearms. The glass filter was fastened to a ring stand. Above the filter an iron ring, 12 cm in diameter, was also fastened to the ring stand. A glass Mooney airvent funnel, 22 cm in diameter, was placed into the iron ring. The funnel was lowered on the ring stand so that its spout would fit inside the glass filter. A test tube with a sidearm was connected to the glass filter. The sidearm was connected by rubber tubing to a trap, and the trap was connected to the vacuum nozzle of a water aspirator. The trap had a valve to open the system to the atmosphere.

The unsprayed, marked plate was positioned in the funnel. Two corners of the plate rested on the inside wall of the funnel. The plate was further secured by allowing it to lean back against the iron rod of the ring stand. Thus, two edges of the plate were horizontal and the plane of the plate lay at about a 60 degree angle with horizontal.

The portion of silica gel G which contained the monoglycerides was scraped from the plate with a wood chisel. The area was scraped as clean as possible. The scraping fell into the funnel and down into the fritted glass filter. When scraping was finished, the scraped area was sprayed with diethyl ether from a 25 ml volumetric

pipet. When no loose scrapings were visible on the scraped area, spraying was directed at the funnel. The funnel was also sprayed clean of loose scrapings.

All scrapings and sprayings fell into the glass filter. When, due to spraying, the level of diethyl ether in the filter rose to 0.5 to 1.0 cm above the top of the scrapings, spraying stopped. The system below the filter was evacuated, and diethyl ether would begin to pass through the filter into the test tube below. When the level of the ether reached the top of the scrapings, the vacuum was released. When all spraying was completed, a vacuum was created below the glass filter until all the diethyl ether was in the test tube. The vacuum hose was then removed from the test tube. The scrapings in the glass filter were discarded and the inside of the filter was rinsed out with diethyl ether. The test tube and its contents were covered and stored. A new tube was connected to the glass filter and to the vacuum system. The scraping and spraying of the silica gel G containing the diglycerides then proceeded.

The technique described above was repeated for each remaining band of silica gel G.

The test tubes containing the six fractions were subjected to mild vacuum at room temperature. When only one milliliter or less of solution remained in a tube, the solution was transferred to a small volumetric flask. The transfer was made quantitative by rinsing the tube with several aliquots of benzene. The benzene was added to the volumetric flask. The contents of the flask were brought up to the mark with additional benzene. The flask was stoppered and inverted

five times.

Six small, tared Teflon cups were placed on a hot plate set at 40°C. Two milliliters of lipid solution from the flask were pipetted into one cup. This process was repeated using another flask and another cup until each of the six cups contained a different one of the six fractions. Roofs of aluminum foil were positioned to prevent dust from falling into the cups. After the solvents had evaporated, the cups were placed in a vacuum oven set at 40°C. The fractions were dried *in vacuo* for one hour. The cups were then placed in a desiccator and were weighed within one hour on a Cahn Gram Electrobalance. The mass of lipid in any fraction was calculated by the equation

$$K = QY/2\ ml, \tag{42}$$

where K is the mass of lipid in a fraction,

Q is the volume of the volumetric flask, and

Y is the mass of the lipid in the Teflon cup.

The beaker from which the lipids were applied to the unsprayed plate was placed on a hot plate set at 40°C. A stream of nitrogen directed into the beaker aided the removal of excess solvent. The beaker and its contents were then dried *in vacuo* at 40°C. The beaker was cooled in the cooling chamber of the Mojonnier Milk Tester for seven minutes. Finally, the beaker was weighed on an analytical balance. The difference between this weighing and the previous weighing was the amount of lipids applied to the thin layer chromatogram.

The results of the quantitative determination of the major classes of neutral lipids of the three pellets are given in Table 15.

The attempt to quantitatively determine the major classes of the polar lipids by the gravimetric analysis. Qualitative determination of the major classes on thin layer chromatograms. Essentially the same techniques used to quantitatively determine the neutral lipids were applied to the polar lipids of the three pellets. Some modifications of technique were necessary. The solvent system used in development of the thin layer chromatograms was chloroform-methanol-water-acetic acid, 80:35:5:1 (v/v/v/v). Following the scraping of bands of silica gel G from the plate, the scraped areas were sprayed with methanol. The methanolic solutions of lipids eluted from the silica gel G were transferred from the test tubes to small round bottom flasks with ground glass joints. The methanol was evaporated *in vacuo* on the Rinco rotary evaporator. The lipids were transferred from the flask to a volumetric flask with several small aliquots of benzene. The remainder of the procedure has been described above.

The above technique did not produce satisfactory results. The percentage recoveries of polar lipids from the TLC plates ranged between 44 and 276%. Why was there such a wide, unsatisfactory range? The major constituents of silica gel G are silicic acid and calcium sulfate. Recall that the polar lipids were eluted from the silica gel G with methanol. It was assumed that the constituents of silica gel G were not soluble in methanol. It was further assumed that these same constituents did not pass through the ultra-fine fritted glass filter during the elution of the polar lipids. One or both of these assumptions had to be wrong. The eluted polar lipids were contaminated in every experiment. Further, it appeared that the polar lipids were bound to the contaminants. When this point became appar-

ent, it was decided to try to break the binding. The most elaborate attempt to break the binding involved a common lipid extraction technique. The lipid-contaminate mixtures isolated from a TLC plate were extracted by the procedure of the Mojonnier fat test. Ammonium hydroxide was omitted from the extraction. Three applications of the Mojonnier technique produced recoveries of 63, 120, and 145%. These results were unsatisfactory.

Figure 6 shows a typical thin layer chromatogram of the polar lipids of a pellet. Five broad bands can be seen. One is at the origin and one is at the solvent front. The remaining three are between the above two.

In identifying the bands in Figure 6 heavy reliance was placed on the literature. From the reports of Wood et al. (1964), Roelofsen et al. (1964), Privett et al. (1965), Abramson and Blecher (1964), Mangold (1961), and Robinson and Phillips (1963), the bands can be quasi-identified from the origin forward as lysolecithin, sphingomyelin, lecithin, cephalin, and the leading band at the solvent front. The finding of sphingomyelin, lecithin and cephalin is in accordance with data given in Table 2.

Following development thin layer chromatograms of the polar lipids were sprayed with the reagent described by Dittmer and Lester (1964). This reagent was reported to be specific for phosphate esters, such as occur in most known phospholipids. Of the five bands only the sphingomyelin, lecithin, and cephalin bands gave positive tests. This result seemed to eliminate the possibility that the band at the origin was the phosphate-containing lysolecithin.

The thin layer chromatograms of the polar lipids were also

sprayed with 0.2% ninhydrin in 95% ethanol. The only positive reaction was given by the cephalin band.

The attempt to quantitatively determine the polar lipids of the three pellets by a gravimetric analysis failed. The use of TLC to fractionate the polar lipids was still desired.

The logical choice of a technique to quantitatively determine the polar lipids was a phosphorus analysis. Robinson and Phillips (1963) separated serum phospholipids on a thin layer chromatogram. The chromatogram was sprayed with 18N sulfuric acid and heated until black carbon was visible. The phosphorus analysis was performed on each entire blackened area, including adsorbent. However, the adsorbent interfered strongly with the colorimetric test.

Skipski et al. (1964) separated phospholipids on a thin layer of silica gel. They exposed the developed TLC plate to iodine vapor. The colored areas were scraped off the plate. The phospholipids were eluted from the adsorbent. A phosphorus determination was made on the eluate from each colored area.

The results reported by Skipski et al. (1964) were excellent and were superior to those reported by Robinson and Phillips (1963).

It was decided that the determination of the polar lipids of the three pellets would follow a procedure which was roughly similar to that of Skipski et al. (1964).

Preparation No. 2

A different ten gallons of milk were collected for preparation No. 2. The methods used in preparation No. 2 resembled those of preparation No. 1 exactly with some exceptions. The exceptions are

explained below.

A De Laval model 518 centrifugal cream separator was used for the separation of the milk into skimmilk and cream and for the washing of the cream. The cream was washed three times.

The 7,500S pellet, the 230S pellet, and the 35S pellet were redispersed and recentrifuged 9, 7, and 6 times, respectively.

The appearances of the three pellets of preparation No. 2 were exactly similar to the appearances of the three pellets of preparation No. 1.

When the supernatant above a pellet was considered sufficiently clear, the pellet was redispersed in the antibiotic solution. The total mass of each pellet suspension was obtained. The per cent total solids of each pellet suspension was obtained by the Mojonnier total solids test. The results of the total solids tests are given in Table 16.

The total solids of each of the three pellets was calculated by an equation similar to equation (40):

$$m = 1230\ M \left[a - (c/d)\right] /980, \qquad (43)$$

where m is the mass of the total solids of a pellet,

1230 ml is the volume of the membrane suspension,

980 ml is the volume of membrane suspension fractionated,

M is the mass of the pellet suspension actually isolated,

a is the mean of four determinations of the per cent total solids of a pellet suspension divided by 100,

c is the concentration of antibiotics in the antibiotic solution, and

d is the density of the pellet suspension and was assumed

to be 1 gram/ml.

Pellet suspension masses, total solids data, and appearances of the 7,500S, 230S, and 35S pellets are given in Table 17.

Extraction of total lipids from the pellet suspensions. Determination of the amount of lipid in each pellet. The amount of lipid in each pellet was calculated by an equation similar to equation (41):

$$L = (1230\ M/980)(1/n)\sum_{i=1}^{n}(h_i/H_i) \quad , \qquad (44)$$

where L, n, h_i, and H_i are defined exactly as in equation (41), and 1230 M/980 is the same as in equation (43).

How h_i and H_i were obtained will now be described.

Each of twelve 250 ml heavy wall glass bottles was tared on the Mettler balance. The twelve bottles were divided into three sets of four bottles per set, one set per pellet suspension. Each pellet suspension was divided more or less equally among the four bottles of a set. Each bottle and its contents were weighed. The difference between the two weighings provided H_i. The pellet suspensions in the twelve bottles were frozen in a dry ice-ethanol bath and were then dried by lyophilization. Following lyophilization 75 ml of methanol were added to each bottle. The bottles were flushed out with nitrogen and stoppered. The bottles were placed on an Eberbach rotary shaker and were shakened for 30 minutes. Following the 30 minutes 75 ml of chloroform were added to each bottle. The bottles which were not immediately used were flushed out with nitrogen, stoppered, and stored at -20°C.

The suspension of chloroform, methanol, and lyophilized pellet in a bottle was filtered through Whatman No. 2 filter paper into a

500 ml glass separatory funnel equipped with a Teflon stopcock.

A small aliquot of methanol was added to the bottle, swirled around, and poured into the filter. This was repeated several times with methanol and then with chloroform. The sums of the volumes of the aliquots of methanol and chloroform were 50 ml and 175 ml, respectively. Seventy-five ml of a 0.01N deionized water solution of potassium chloride were added directly, unfiltered, to the separatory funnel.

The lipids were obtained from the separatory funnel and weighed in exactly the manner explained for preparation No. 1. Values of h_i, H_i, $100h_i/H_i$, and $(100/n)\sum_{i=1}^{n}(h_i/H_i)$ are given in Table 18.

Using the values of M given in Table 17 and the data given in Table 18, L was calculated for each pellet. Values of L and associated data are given in Table 19.

Immediately following the weighing of the lipids, the lipids were dissolved in chloroform and were stored under nitrogen at -20°C.

Separation of the polar lipids from the neutral lipids. The separation of the total lipids into neutral and polar lipids was accomplished in a different manner for preparation No. 2 than for preparation No. 1. The silicic acid column technique of Ways and Hanahan (1964) requires a great deal of time. For want of a better technique, the principle of the silicic acid column was incorporated into a method which was faster than the column technique. The principle, of course, is that in the presence of an excess of chloroform the polar lipids remain strongly bound to heat-activated silicic acid while the neutral lipids are hardly bound at all. When the chloroform is separated from the silicic acid, the neutral lipids are

found in the chloroform and the polar lipids remain with the silicic acid. Methanol is usually used to strip the polar lipids from the silicic acid.

Ways and Hanahan (1964) established a ratio of 20 grams of silicic acid per gram of total lipids to be fractionated. This ratio was adopted for our work. The masses of total lipids to be separated ranged from 1.0 to 1.8 grams. Accordingly, the amounts of silicic acid used ranged from about 20 to 40 grams.

A suction flask was attached to a trap which was attached to a water aspirator. A large, medium-fritted glass filter was fitted to the flask. The appropriate amount of silicic acid was weighed out and transferred into the filter. The silicic acid was washed first with methanol and then with chloroform. The silicic acid was transferred to an aluminum dish and stored overnight at 110^{o}C in a hot air oven.

The following morning the silicic acid was taken from the oven and allowed to cool to room temperature. Simultaneously, the lipid sample was allowed to warm to room temperature. One half of the sample was poured into one 250 ml glass centrifuge bottle, A, and the other half into another, B. The silicic acid was similarly divided and poured dry into the two bottles. Chloroform was added to both bottles until the level of the solvent coincided with the surface of the silicic acid. The contents of both bottles were stirred gently but thoroughly with a glass rod. Bottle A was then filled with chloroform and again stirred thoroughly. It was centrifuged briefly in the International Centrifuge. The supernatant was transferred to bottle B. Bottle A was again filled with chloroform. This time the

contents of both bottles were stirred. Both bottles were centrifuged briefly. The supernatant from Bottle B was passed through an ultra-fine fritted glass filter and collected in a suction flask. The supernatant from A was transferred to B. A was again filled with chloroform. Stirring and centrifuging of the bottles followed. The supernatant from B was filtered and collected as before. The supernatant from A was transferred to B. Stirring the contents of B, centrifuging, filtering and collecting the supernatant, filling B with chloroform, stirring, centrifuging, and filtering and collecting the supernatant followed.

The collected chloroform solution was discarded. A new suction flask was attached to the ultra-fine filter.

The procedure just described was repeated with several modifications. Methanol was used instead of chloroform. B was filled with methanol four times. After each filling the mixture in B was stirred thoroughly and centrifuged. Each supernatant from B was transferred to A, whereupon the contents of A were stirred and centrifuged. Each of the four supernatants from A was passed through the ultra-fine filter and collected. After the fourth supernatant had been poured into the filter, A was filled with fresh methanol; the mixture was stirred, centrifuged, and the supernatant was filtered and collected.

The methanolic solution was transferred to two one-liter round bottom flasks with ground glass joints. The solutions were evaporated *in vacuo* to near dryness on the Rinco rotary evaporator. The contents of the flasks were transferred to a 100 ml volumetric flask with the aid of a succession of several aliquots of methanol, one aliquot of benzene, and several more aliquots of methanol. The solution in the

volumetric flask was made up to the mark by the addition of methanol.

Quantitative determination of the major classes of the phospholipids of the three pellets by TLC and phosphorus analysis. The colorimetric determination of phosphorus was performed by the method of Sumner (1944). Modifications of Sumner's method will now be described. The addition of 5 ml of 7.5N sulfuric acid was omitted. The final volume of a solution to be read in the colorimeter was one of 25, 50, or 100 ml. When the final volume was 25 ml, the amounts of the reagents used by Sumner were divided by two for our use. When the final volume was 100 ml, the amounts were multiplied by two. The final solutions were made up to volume in volumetric flasks with ground glass stoppers. The per cent transmission of each solution was read in a Cenco Photelometer equipped with a red filter (620 mμ).

Phosphorus occurs in phospholipids in the form of phosphate esters. (Exceptions to this statement have been reported by Rouser et al., 1965. It is probable that many more such exceptions will be reported in the future.) The colorimetric test requires that the phosphorus be in the form of orthophosphate. The conversion of phospholipid phosphorus to orthophosphate was accomplished by wet digestion.

Wet digestion was carried out in Pyrex test tubes. The use of other vessels will be described later. Heat for digestion was provided through a sand bath from a hot plate. Phospholipids for digestion were dissolved in methanolic or benzene and methanol solutions. A known volume of solution was pipetted into the Pyrex tube. The tube was placed into the sand bath and the organic solvents were removed by evaporation. As will be seen later, some phospholipid

samples were eluted from a fritted glass filter into the Pyrex tubes.

Standards of deionized water solutions of potassium dihydrogen phosphate were taken through the digestion procedure. In these cases it was not necessary to remove the solvent, water. Blank tubes, containing nothing at the outset, were also subjected to wet digestion.

Ten normal sulfuric acid was added to all digestion vessels. The amount of acid added depended on the final volume of the sample prior to colorimetric determination. 3.75, 7.5, and 15.0 ml of acid were added to samples with final volumes of 25, 50, and 100 ml, respectively.

All vessels were placed in the sand bath. The temperature of each digesting solution was maintained slightly below the boiling point. After three hours of heating, all solutions were heated vigorously until profuse fuming of the acid was observed. When a solution began to fume, it was removed from the sand bath and allowed to cool. When the solutions had cooled to room temperature, four drops of hydrogen peroxide were added to each vessel. The vessels were returned to the sand bath. Additional hydrogen peroxide was added where necessary but only after the vessels were again cooled. When no carbon could be seen in a solution and when the solution was crystal clear and fuming, heating was continued for an additional one and one-half hours. During this time the temperature of the digesting solution was adjusted so that fuming was just barely observable. The one and one-half hours of heating were necessary to destroy the hydrogen peroxide. The contents of each digestion vessel were quantitatively transferred to a volumetric flask with the aid of deionized water. The remainder of the procedure is described

by Sumner (1964).

The concentration of orthophosphate (and phosphorus) in a final solution is related to the per cent transmission of the solution by Beer's Law:

$$c = k \left[2 - \log_{10}(\%T)\right] , \tag{45}$$

where c is the concentration of phosphorus in the final solution,

k is a constant, and

%T is the per cent transmission.

Now, c is equal to the mass of phosphorus, m, divided by the final volume, V, which contains m. That is,

$$c = m/V \tag{46}$$

Substitution of m/V for c in equation (45) gives

$$m = kV \left[2 - \log_{10}(\%T)\right] . \tag{47}$$

Beer's Law is only an empirical expression and must be tested in each case. Phosphorus determinations were made on a series of standards containing from 0 to 0.16 milligrams of phosphorus. The results are given in Table 20 and plotted in Figure 7.

The plot in Figure 7 shows that a straight line is a good approximation for the locus of the plotted points. Thus, $\log_{10}(\%T)$ is nearly a linear function of m and this fact agrees with Beer's Law as expressed in equation (47). Finally, therefore, Beer's Law holds for the colorimetric determination of phosphorus described above for the values of m and V given in Table 20. However, as equation (47) shows, the range of m is extended proportionally when V becomes 50 or 100 ml.

Let

$$2 - \log_{10}(\%T) = OD \tag{48}$$

for the remaining discussion. Equation (47) becomes, therefore,

$$m = kV(OD) \ . \qquad (49)$$

The concentration of phosphorus in the 100 ml of benzene and methanol solution of polar lipids of the 230S pellet is given in Table 21. The same data for the polar lipids of the 7,500S and 35S pellets is given in Table 22.

Thin layers of silica gel G on glass plates 20 cm square were prepared as described for preparation No. 1. The solvent system used for development of the thin layer chromatograms was chloroform-methanol-water-acetic acid, 80:35:5:1 (v/v/v/v). After one development of the TLC plates with the above solvent system, the silica gel G became somewhat unstable, and flaking off of the adsorbent was sometimes observed. It was decided, therefore, not to predevelop the TLC plates prior to chromatographing the polar lipids.

Five TLC plates were prepared simultaneously. Their edges were scraped and the plates were activated by heating as described for preparation No. 1. During the time activation was proceeding a known volume, 2 or 3 ml, of a benzene and methanol solution of the polar lipid of a pellet was pipetted into each of four small glass beakers.

Following activation the TLC plates were removed from the hot air oven and placed into a desiccator.

The lipid solution in each beaker was concentrated to one milliliter or less by heating the beaker at 50°C on a hot plate.

When a TLC plate was cooled to room temperature, the lipids from one beaker were banded on it as described in the TLC of the neutral lipids. Finally, the lipids of each one of the four beakers

were banded to a different one of the best four TLC plates of the original five. Immediately after the lipids were applied to a plate, the plate was stored under nitrogen until the lipids were applied to the remaining plates. All four plates were developed simultaneously. Following development the four plates were allowed to dry. When dry, two of the plates were stored in a desiccator at room temperature. The remaining two plates were sprayed lightly with concentrated sulfuric acid. The two plates were heated at 110^{o}C in a hot air oven until black carbon bands became visible. The two unsprayed plates were marked with a wax pencil according to the locations of the bands on the sprayed plates.

The recovery of the lipids from the two unsprayed plates was carried out in the same general manner as described for preparation No. 1. The modifications which were made in the technique will now be described. Methanol was used to elute the polar lipids from the silica gel G. During the elution process the test tube used to catch the eluate was immersed in a hot water bath. The temperature of the bath was maintained at 95^{o}C through use of a hot plate. This system accomplished almost instant evaporation of methanol from the eluate.

Wet digestion of the polar lipids was carried out in the test tubes used to catch the eluate.

Wet digestion was also carried out in the two beakers from which lipids were applied to the two unsprayed plates.

Standards and blanks were digested concurrently with the lipid samples.

The objective of this work was to determine what percentage

of the total phosphorus recovered from a TLC plate was the recovered phosphorus of one of the major classes of polar lipids. Equation (49) shows that OD is directly proportional to m. It was possible, therefore, to obtain the stated objective through a calculation using OD as the basis, or extensive variable, providing V was a constant for all fractions involved in the calculation. This is what was done.

No calculations of the percentage recovery of phosphorus from the TLC plates were attempted. Recall that the contents of the beakers from which lipids were applied to the unsprayed plates were later subjected to wet digestion and colorimetric determination. Because of the shortness in height of the beakers, the beakers were poorer digestion vessels than were test tubes. It is quite likely that some digestion solution was systematically lost from beakers during digestion. This seemed to be the case especially following the addition of hydrogen peroxide. The bubbling and release of gaseous oxygen from the beaker was probably accompanied by a loss of phosphorus. Percentage recovery of phosphorus would usually be expressed in the following way.

$$\% \text{ recovery} = \frac{(100)\ \text{Phosphorus recovered from TLC plate}}{\text{Phosphorus in beaker originally} - \text{Phosphorus recovered from beaker}} . \qquad (50)$$

The amount of polar lipids applied to a TLC plate was usually something less than half of the amount in the beaker. Thus, a loss of phosphorus from the beaker would greatly affect the percentage recovery expressed by equation (50).

The per cent recovery of phosphorus from the beaker was calculated by a modification of equation (50).

$$\% \text{ recovery} = (100)\frac{\text{Phosphorus recovered from TLC plate} + \text{Phosphorus recovered from beaker}}{\text{Phosphorus in beaker originally}}, \quad (51)$$

where $\text{Phosphorus recovered from TLC plate} = k\left[V(OD)\right]_{\text{total of plate}}$,

$\text{Phosphorus recovered from beaker} = k\left[V(OD)\right]_{\text{beaker}}$, and

$\text{Phosphorus originally in beaker} = Z(\text{Volume of solution placed in beaker})$,

where Z is defined in Tables 21 and 22.

The relative amounts of phosphorus in the major classes of polar lipids as well as the per cent recoveries are given in Tables 23 through 30.

Table 6 The minimum sedimentation coefficient of a pellet and the time required to sediment it at 21,000 rpm in the Type 21 Rotor

Minimum sedimentation coefficient (S)	Time (min)
400	64.5
80	323
40	645

Table 7 Results of Mojonnier fat tests and total solids tests performed on a membrane suspension and on the 400S, 80S, and 40S supernatants

Fraction	Wt % solids	Wt % lipids
Membrane suspension	2.11	1.544
400S supernatant	0.17	0.073
80S supernatant	0.09	0.036
40S supernatant	0.063	0.017

Table 8 Separation of an aqueous suspension of the fat globule membrane into three pellets by differential sedimentation in the ultracentrifuge at $0^{o}C$ Conditions of centrifugation and sedimentation coefficients of the pellets

Rotor speed (rpm)	Length of time of centrifuge run (min)	Minimum sedimentation coefficient of pellet (S)
8,500	20	7,500
21,000	110	230
21,000	720	35

Table 9 Preparation No. 1--Per cent total solids of 7,500S, 230S, and 35S pellet suspensions Analysis by Mojonnier total solids test

Pellet suspension	i	Per cent total solids: Individual determination	Per cent total solids: Mean value for pellet suspension
7,500S	1	0.771	0.774
	2	0.772	
	3	0.779	
230S	1	0.659	0.660
	2	0.661	
	3	0.659	
35S	1	0.28	0.27
	2	0.26	
	3	0.29	
	4	0.25	

Table 10 Preparation No. 1--Pellet suspension masses, pellet suspension densities, total solids data, and appearances of the 7,500S, 230S, and 35S pellets

Pellet	Mass of pellet suspension (g)	Density of pellet suspension (g/ml)	Total solids of pellet (g)	Per cent of sum of total solids	Appearance
7,500S	524.7	0.99908	5.30	45.2	white
230S	639	0.99783	5.50	46.9	white and brown
35S	271.0	0.99746	0.93	7.9	brown
Sum			11.73		

Table 11 Amount of lipid extracted from a 7,500S pellet suspension A comparison of four different procedures of extracting lipids

Procedure	i	$\frac{\text{Mass of lipid extracted from suspension}}{\text{Mass of suspension extracted}}(100)$ (%)	Mean value for each procedure (%)
Mojonnier	1	0.352	0.356
	2	0.360	
Mojonnier without NH_4OH	1	0.425	0.426
	2	0.424	
	3	0.428	
Mojonnier: aqueous 0.67N KCl substituted for NH_4OH	1	0.432	0.434
	2	0.436	
Chloroform methanol	1	0.439	0.439

Table 12 Preparation No. 1--Amount of lipid in each of the 7,500S, 230S, and 35S pellets (see equation 41)

Pellet	i	$100h_i/H_i$ (%)	$(100/n)\sum_{i=1}^{n}(h_i/H_i)$ (%)	L (g)	Percentage of sum of L's (%)
7,500S	1	0.5392	0.5354	3.70	49.7
	2	0.5316			
230S	1	0.381	0.382	3.22	43.2
	2	0.383			
35S	1	0.147	0.148	0.528	7.1
	2	0.148			
Sum				7.45	

Table 13 Preparation No. 1--Percentage lipid of pellet total solids (see Tables 10 and 12)

Pellet	$\frac{\text{Mass of pellet lipids}}{\text{Mass of pellet total solids}}(100)$ (%)
7,500S	69.8
230S	58.5
35S	57
7,500S + 230S + 35S	63.5

Table 14 Preparation 1--Results of the separation of the total lipids of each of the three pellets into neutral lipids and polar lipids by silicic acid column chromatography

Pellet	i	Mass of total lipids applied to column (g)	a, the mass of lipids recovered from column (g)	Percentage recovery	b, the mass of neutral lipids recovered (g)	100b/a (%)	c, the mass of polar lipids recovered (g)	100c/a (%)
7,500S	1	0.1164	0.1220	104.8	0.0836	68.5	0.0384	31.5
	2	0.0960	0.0995	104	0.0692	69.5	0.0303	30.5
230S	1	0.0877	0.0891	102	0.0549	61.6	0.0342	38.4
35S	1	0.0348	0.0355	102	0.0206	58.0	0.0149	42.0

Table 15 Preparation No. 1--Amounts of the major classes of neutral lipids in the 7,500S, 230S, and 35S pellets

	7,500S pellet		230S pellet		35S pellet	
	Mass recovered from TLC plate (mg)	Percentage of total	Mass recovered from TLC plate (mg)	Percentage of total	Mass recovered from TLC plate (mg)	Percentage of total
Monoglycerides	0.92	4.4	0.29	2.3	1.90	26
Diglycerides	0.72	3.4	0.21	1.7	1.22	17
Cholesterol	1.13	5.3	1.36	10.8	0.83	11
Fatty acids	3.04	14.3	1.93	15.3	1.37	19
Triglycerides	14.94	70.5	8.60	68.0	1.85	26
"Leading band"	0.45	2.1	0.25	2.0	0.0	0
Total	21.20		12.64		7.17	
Mass of lipid applied to TLC plate (mg)	19.9		11.7		6.6	
Recovery (%)	107		108		109	

Table 16 Preparation No. 2--Per cent total solids of 7,500S, 230S, and 35S pellet suspensions (Analysis by Mojonnier total solids test)

		Per cent total solids	
Pellet suspension	i	Individual determination	Mean value for pellet suspension
7,500S	1	1.10	1.09
	2	1.07	
	3	1.07	
	4	1.10	
230S	1	1.27	1.27
	2	1.26	
	3	1.28	
	4	1.28	
35S	1	0.39	0.37
	2	0.35	
	3	0.37	
	4	0.38	

Table 17 Preparation No. 2--Pellet suspension masses, total solids data, and appearances of the 7,500S, 230S, and 35S pellets

Pellet	M, the mass of a pellet suspension (g)	Total solids of pellet (g)	Per cent of sum of total solids	Appearance
7,500S	430.62	5.84	33.8	white
230S	567.68	8.97	51.8	white and brown
35S	558.42	2.5	14.0	brown
Sum		17.3		

Table 18 Preparation No. 2--Per cent lipids of the 7,500S, 230S, and 35S pellets (see equation 44)

Pellet	i	H_i (g)	h_i (g)	$\frac{100h_i}{H_i}$ (%)	$\frac{100}{n}\sum_{i=1}^{n}\frac{h_i}{H_i}$ (%)
7,500S	1	103.76	0.5064	0.4880	0.4989
	2	103.16	0.5197	0.5038	
	3	98.94	0.4994	0.5048	
230S	1	125.66	0.9665	0.7691	0.7607
	2	148.92	1.1153	0.7489	
	3	149.90	1.1629	0.7758	
	4	133.00	0.9961	0.7489	
35S	1	131.86	0.2424	0.1838	0.1838
	2	133.24	0.2449	0.1838	
	3	152.48	0.2802	0.1838	

Table 19 Preparation No. 2--Mass of lipid in each of the 7,500S, 230S, and 35S pellets, and associated data

Pellet	L, mass of lipid (g)	Percentage of sum of L's	$\frac{100(\text{mass of lipid in pellet})}{\text{mass of solids in pellet}}$ (%)
7,500S	2.697	28.68	46.2
230S	5.419	57.62	60.4
35S	1.289	13.71	52
7,500S +230S +35S	9.405		54.4

Table 20 Application of phosphorus determination to a series of standards[a] (see equations 45 through 47)

m, the mass of phosphorus in standard (mg)	% T	$\log_{10}$(% T)
0.00	100.0	2.00
0.01	84.0	1.92
0.02	72.0	1.86
0.04	54.6	1.74
0.08	29.0	1.46
0.16	9.0	0.95

[a]Final volume of each standard was 25 ml

Table 21 Preparation No. 2--Determination of Z, the mass of phosphorus in 1 ml of the benzene and methanol solution of polar lipids of the 230S pellet (see equations 45-49)

Sample	i	V (ml)	% T	Mean of % T	OD	k	m (mg)	Z (mg/ml)
Standard 0.16 mg phosphorus	1	50	33.6	34.0	0.469	$\frac{0.16}{50(0.469)}$		
	2	50	33.8					
	3	50	33.8					
	4	50	34.8					
1 ml of polar lipids of 230S pellet	1	50	26.0	25.2	0.599	$\frac{0.16}{50(0.469)}$	0.204	0.204
	2	50	25.0					
	3	50	24.8					
	4	50	25.0					

Table 22 Preparation No. 2--Determination of Z, the mass of phosphorus in 1 ml of each of the benzene and methanol solutions of polar lipids of the 7,500S and 35S pellets (see equations 45-49)

Sample	V (ml)	% T	OD	k	m (mg)	Z (mg/ml)
Standard 0.16 mg phosphorus	50	30.4	0.517	$\frac{0.16}{50(0.517)}$		
0.5 ml of polar lipids of 7,500S pellet	50	45.0	0.347	$\frac{0.16}{50(0.517)}$	0.108	0.215
1 ml of polar lipids of 35S pellet	50	36.8	0.434	$\frac{0.16}{50(0.517)}$	0.134	0.134

Table 23 Preparation No. 2--Phosphorus analysis of the polar lipids of the 7,500S pellet Determination 1

		V (ml)	% T	OD	k	$\frac{OD}{\text{Total OD}}(100)$ (% phosphorus of total phosphorus)
	Standard 0.08 mg phosphorus	25	33.8	0.471	$\frac{0.08}{25(0.471)}$	
	"Lysolecithin"	25	98.2	0.008		1.0
	Sphingomyelin	25	64.8	0.188		26.8
	Lecithin	25	47.0	0.328		46.7
	Cephalin	25	66.4	0.178		25.4
	Leading band	25	100.0	0.000		0.0
Total				0.702		
	beaker	100	39.5	0.403		

Table 24 Preparation No. 2--Phosphorus analysis of the polar lipids of the 7,500S pellet Determination 2

	V (ml)	% T	OD	k	$\frac{OD}{\text{Total OD}}(100)$ (% phosphorus of total phosphorus)
Standard 0.08 mg phosphorus	25	33.8	0.471	$\frac{0.08}{25(0.471)}$	
"Lysolecithin"	25	100.0	0.000		0.0
Sphingomyelin	25	57.8	0.238		32.6
Lecithin	25	49.0	0.310		42.5
Cephalin	25	65.8	0.182		24.9
Leading band	25	100.0	0.000		0.0
Total			0.730		
beaker	100	40.0	0.398		

Table 25 Preparation No. 2--Phosphorus analysis of the polar lipids of the 230S pellet Determination 1

	V (ml)	% T	OD	k	$\frac{OD}{\text{Total OD}}(100)$ (% phosphorus of total phosphorus)
Standard 0.08 mg phosphorus	25	38.5	0.415	$\frac{0.08}{25(0.415)}$	
"Lysolecithin"	25	98.6	0.006		0.7
Sphingomyelin	25	59.2	0.228		27.2
Lecithin	25	43.2	0.365		43.6
Cephalin	25	57.8	0.238		28.4
Leading band	25	99.8	0.001		0.1
Total			0.838		
beaker	100	45.0	0.347		

Table 26 Preparation No. 2--Phosphorus analysis of the polar lipids of the 230S pellet Determination 2

		V (ml)	% T	OD	k	$\frac{OD}{\text{Total OD}}(100)$ (% phosphorus of total phosphorus)
	Standard 0.08 mg phosphorus	25	38.5	0.415	$\frac{0.08}{25(0.415)}$	
	"Lysolecithin"	25	99.2	0.003		0.7
	Sphingomyelin	25	76.0	0.119		27.2
	Lecithin	25	61.2	0.213		48.7
	Cephalin	25	79.0	0.102		23.3
	Leading band	25	100.0	0.000		0.0
Total				0.437		
	beaker	100	39.0	0.409		

Table 27 Preparation No. 2--Phosphorus analysis of the polar lipids of the 35S pellet Determination 1

	V (ml)	% T	OD	k	$\frac{OD}{\text{Total OD}}(100)$ (% phosphorus of total phosphorus)
Standard 0.08 mg phosphorus	25	34.8	0.458	$\frac{0.08}{25(0.458)}$	
"Lysolecithin"	25	100.0	0.000		0.0
Spningomyelin	25	75.3	0.123		40.9
Lecithin	25	80.6	0.094		31.2
Cephalin	25	82.5	0.084		27.9
Leading band	25	100.0	0.000		0.0
Total			0.301		
beaker	100	33.0	0.481		

Table 28 Preparation No. 2--Phosphorus analysis of the polar lipids of the 35S pellet Determination 2

	V (ml)	% T	OD	k	$\frac{OD}{\text{Total OD}}(100)$ (% phosphorus of total phosphorus)
Standard 0.08 mg phosphorus	25	34.8	0.458	$\frac{0.08}{25(0.458)}$	
"Lysolecithin"	25	97.0	0.013		5.2
Sphingomyelin	25	76.0	0.119		47.4
Lecithin	25	86.6	0.062		25.0
Cephalin	25	87.7	0.057		23.0
Leading band	25	100.0	0.000		0.0
Total			0.251		
beaker	100	30.0	0.523		

Table 29 Mean values of 100(OD)/total OD of two determinations of the polar lipids of each of the 7,500S, 230S, and 35S pellets (see Tables 23 through 28)

	(% phosphorus of total phosphorus)		
	7,500S	230S	35S
"Lysolecithin"	0.5	0.7	2.6
Sphingomyelin	29.7	27.2	44.1
Lecithin	44.6	46.2	28.1
Cephalin	25.2	25.9	25.4
Leading band	0.0	0.0	0.0

Table 30 Preparation No. 2--Percentage recovery of phosphorus as expressed in equation (51) for each determination of polar lipids of the 7,500S, 230S, and 35S pellets

Pellet	Determination number	Recovery (%)
7,500S	1	91.2
	2	91.5
230S	1	105.0
	2	97.8
35S	1	96.7
	2	102.0

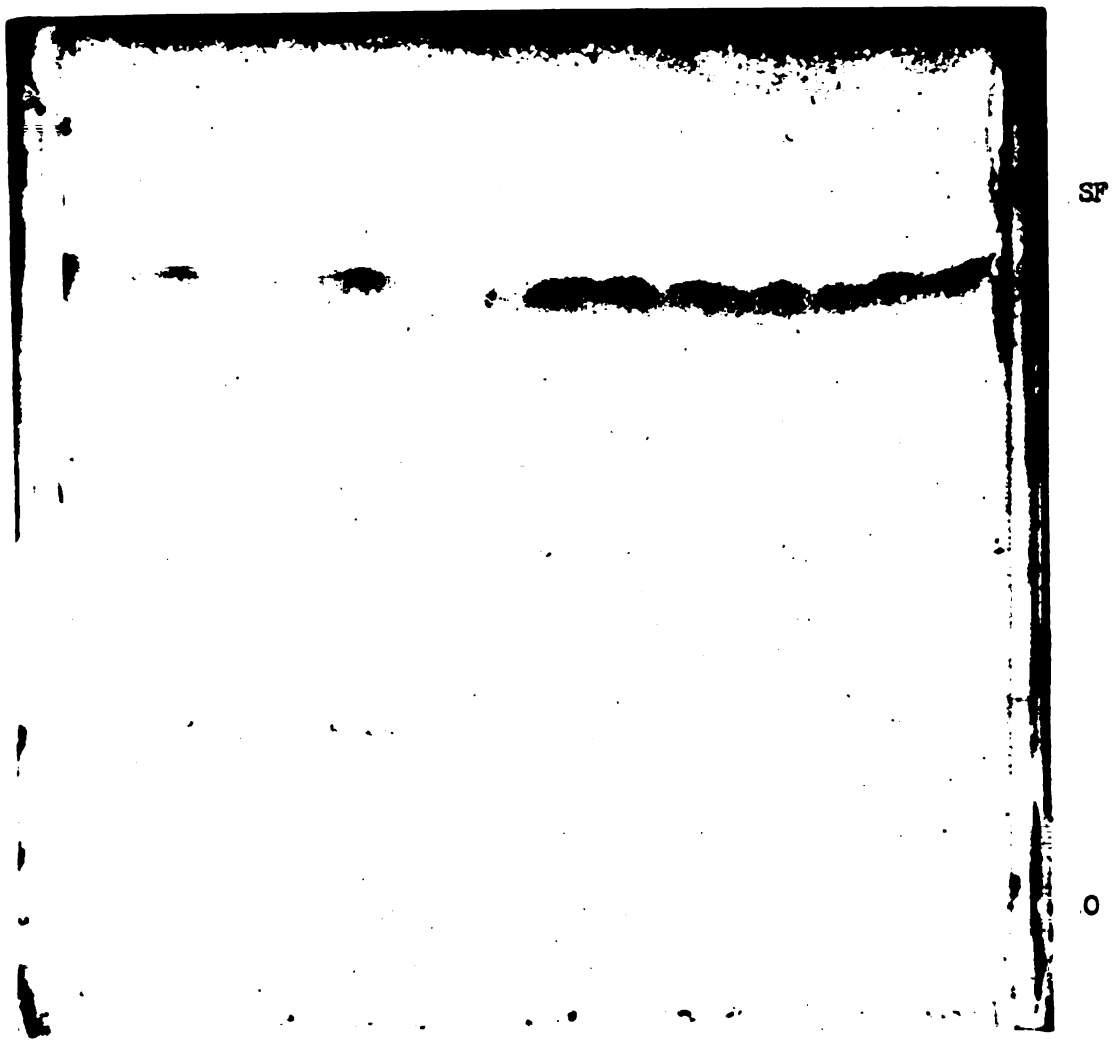


Figure 3 Photograph of a thin layer chromatogram of the neutral lipids of the 7,500S pellet Developing solvent: hexane-diethyl ether-acetic acid, 79:21:1 (v/v/v) Bands were made visible by charring with concentrated sulfuric acid SF and O show the locations of the solvent front and origin, respectively

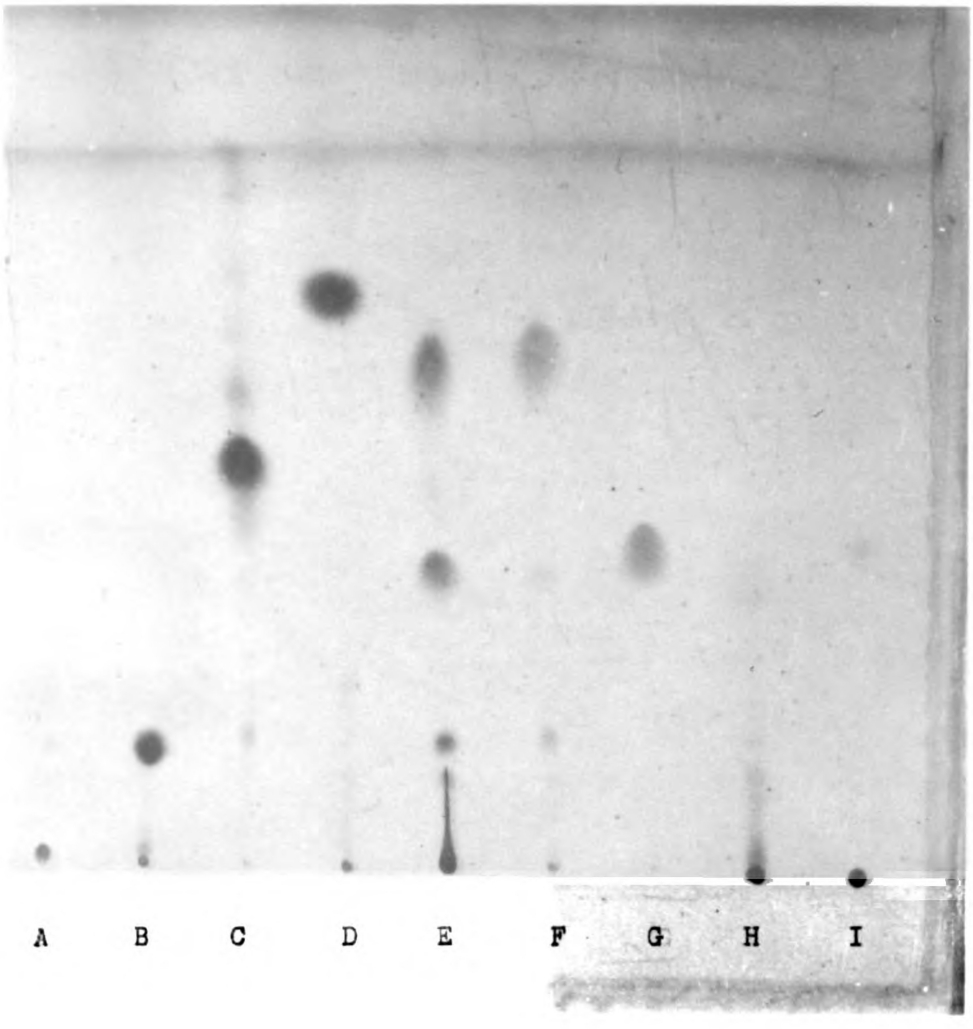


Figure 4 Photograph of a thin layer chromatogram Developing solvent: hexane-diethyl ether-acetic acid, 79:21:1 (v/v/v) The spots were made visible by charring with concentrated sulfuric acid From left to right the samples are: A, Myverol monoglycerides; B, cholesterol; C, alpha-tocopherol; D, cholesterol acetate; E, 7,500S pellet total lipids; F, hydrogenated tallow; G, stearic acid; H, cephalin; and I, beta carotene

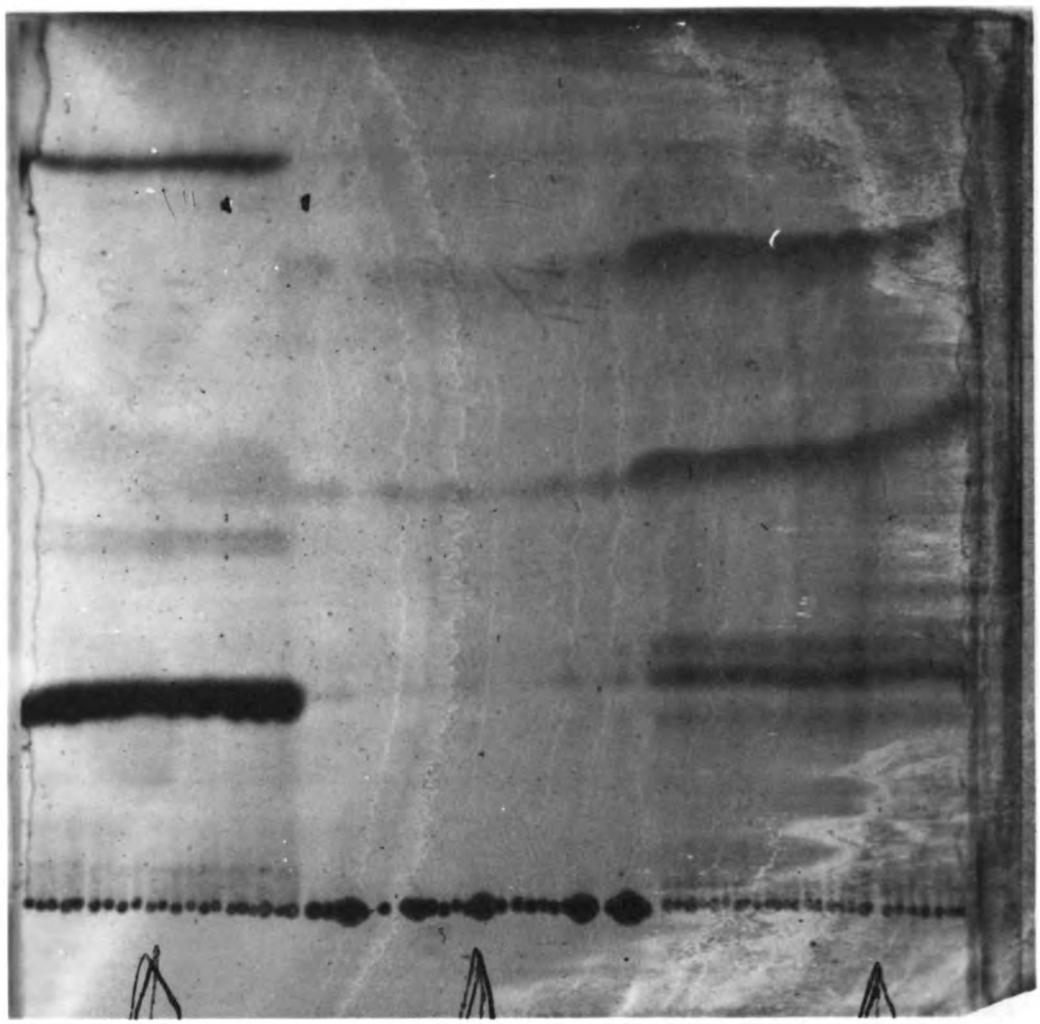


Figure 5 Photograph of a thin layer chromatogram Developing solvent: hexane-diethyl ether-acetic acid, 73:25:2 (v/v/v) Bands were made visible by charring with concentrated sulfuric acid Samples: left one-third, reaction mixture containing cholesterol and stearic acid; middle one-third, membrane suspension total lipids; right one-third, 7,500S pellet neutral lipids SF shows the location of the solvent front

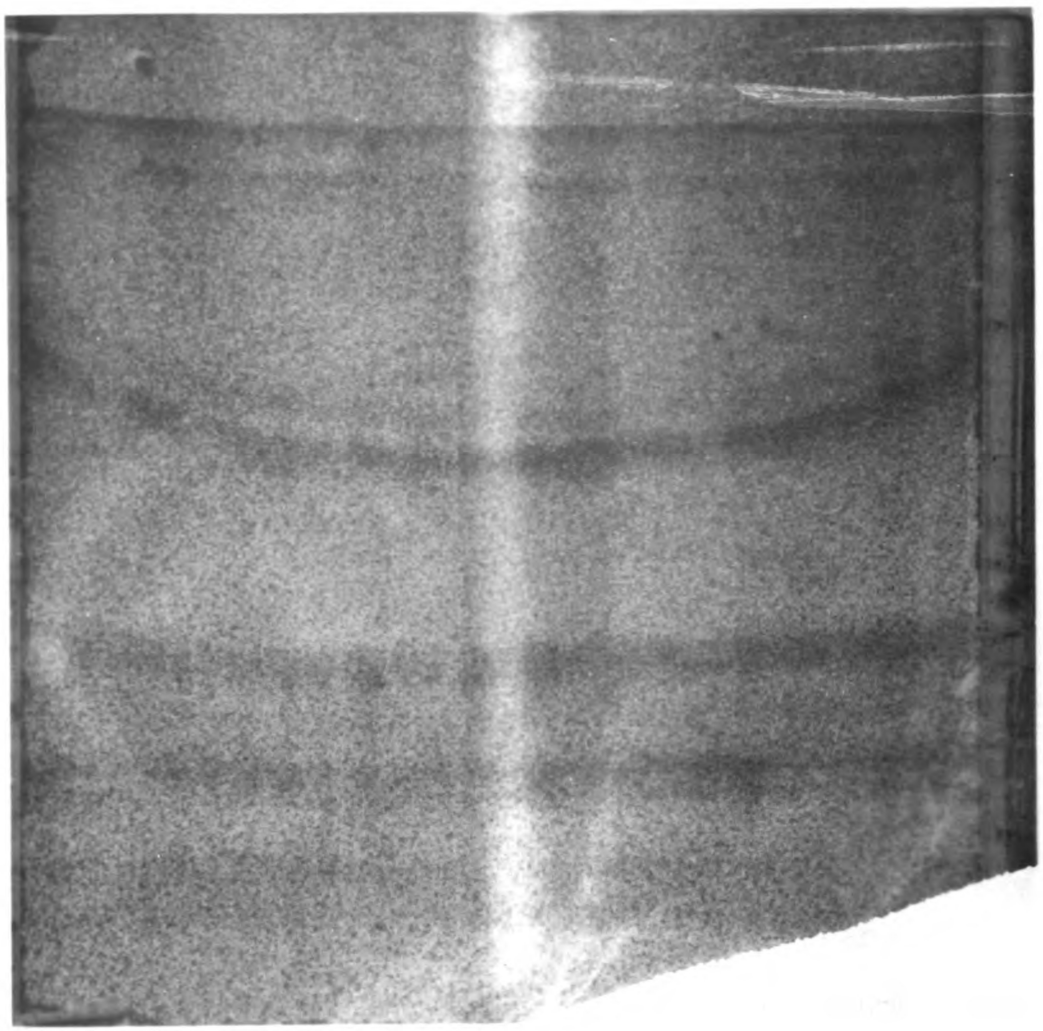


Figure 6 Photograph of a thin layer chromatogram of the 7,500S pellet polar lipids Developing solvent: chloroform-methanol-water-acetic acid, 80:35:5:1 (v/v/v/v) Bands were made visible by charring with concentrated sulfuric acid SF and O show the locations of the solvent front and origin, respectively

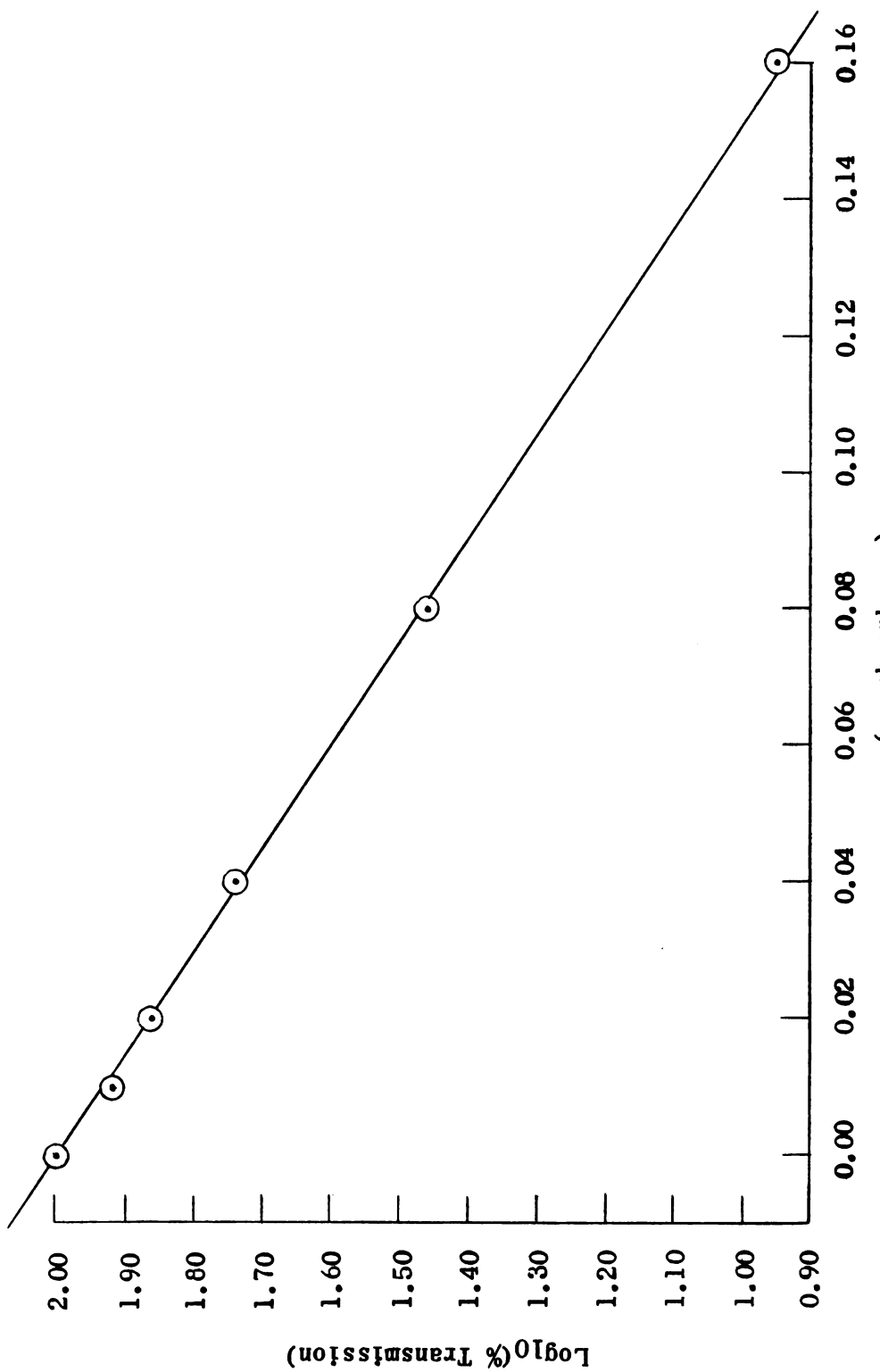


Figure 7 A plot of $\log_{10}$(% T) vs m (see Table 20)

DISCUSSION

The separations of aqueous suspensions of the fat globule membrane of cows' milk into 7,500S, 230S, and 35S pellets were successful. Each pellet was obtained in sufficient quantity for the required analyses. The appearance of each pellet was distinctive from the others.

R, the sum of the total solids of the three pellets recovered from one liter of milk can be expressed by the following aquation:

$$R = \frac{0.26417762 \text{ gallon}}{1 \text{ liter}} (P) , \qquad (52)$$

where P is the sum of the total solids of the three pellets recovered from approximately ten gallons of cows' milk.

For preparation No. 1, P is given in Table 10 as 11.73 grams. For preparation No. 2, P is given in Table 17 as 17.3 grams. By the use of equation (52) values of R for preparations No. 1 and No. 2 were calculated as 0.3099 g/l and 0.457 g/l, respectively. These values of R are in the range of the value of the total membrane solids given by King (1955), 0.35 g/l. Both values, however, are greater than the amount, 0.25 g/l, of "milk microsomes" recovered from cows' milk by Morton (1954).

The distribution of the total solids among the three pellets in each of preparation No. 1 and preparation No. 2 can be seen in Tables 10 and 17, respectively. In each preparation the 230S pellet contained more total solids than either of the other two pellets. In preparation No. 1, however, the masses of the total solids of the 7,500S and 230S pellets were nearly similar. The 35S pellet in each

preparation contained a small fraction of the sum of the total solids of the three pellets.

The data given in Tables 12 and 18 provide some evidence that the chloroform-methanol technique used in the extraction of the total lipids of each pellet was a fairly reliable analytical technique from the standpoint of reproducibility.

The distribution of the total lipids among the three pellets in each of preparation No. 1 and preparation No. 2 can be seen in Tables 12 and 19, respectively. The similarity of the data between the two preparations is only slight. In preparation No. 2 the 230S pellet contained twice as much lipid as the 7,500S pellet. In preparation No. 1 the 7,500S pellet contained slightly more lipid than the 230S pellet. The 35S pellet in each preparation contained a small fraction of the sum of the total lipids of the three pellets. It can be noted that the fraction just mentioned was nearly the same as the fraction, 35S pellet total solids divided by the sum of total solids of the three pellets, for each preparation.

The percentage of pellet total solids which is lipid for each of preparation No. 1 and preparation No. 2 can be seen in Tables 13 and 19, respectively. The values marked "7,500S + 230S + 35S," 63.5% for preparation No. 1 and 54.4% for preparation No. 2, can be compared with similar data reported by other workers. As shown in Tables 3 and 4, Thompson *et al.* (1961) reported two values for the percentage of the fat globule membrane which is lipid, 67.51% and 43.76%. The REVIEW OF THE LITERATURE gives the values reported by Alexander and Lusena (1961), 60.2%, and by Sander (1962), 47%. Richardson and Guss (1965) reported that the fat globule membrane consisted of 48.1%

lipid, as shown in Table 5. Thus, the values reported here for preparations No. 1 and No. 2 are consistent with the values reported by other workers.

Table 14 reports the results of the separation of the total lipids of the three pellets into neutral and polar lipids. Comparison of the data in the column marked "100c/a" with the data in Table 1 points out a salient quality of the fat globule membrane. The concentration of polar lipids in the total lipids of the membrane is much higher than in the whole milk lipid fraction. This is not surprising in view of the work of Mulder et al. (1957), who reported that the majority of the phospholipids in whole milk are located on the fat globule membrane.

In Table 14 the value of 100c/a for the 35S pellet is slightly greater than that for the 230S pellet. Both values are significantly greater than the value of 100c/a for the 7,500S pellet. This result can be expressed in the following way: the data show that the more vigorous the conditions of centrifugation are for sedimenting a particular pellet, the higher is the percentage of the pellet's total lipids that are polar lipids. This result agrees with the data of Alexander and Lusena (1961). Their results have been summarized in the REVIEW OF THE LITERATURE. The above result does not, however, agree with the reports of Brunner (1962) or of Richardson and Guss (1965).

Table 15 gives the amounts of the major classes of neutral lipids in the 7,500S, 230S, and 35S pellets. The per cent recoveries of neutral lipids from the TLC plates are greater than what would be desired. The recoveries are reasonable, however, in light of the

results of similar work by other workers. Komarek et al. (1964) reported per cent recoveries of bovine semen lipids from TLC plates ranging from 86.2 to 101.0%. The per cent recoveries reported by Levin and Head (1965) ranged from 96 to 118%.

The distribution of major classes of neutral lipids in the 230S pellet was quite similar to that of the 7,500S pellet. The distribution of the major classes in the 35S pellet, however, was radically different from the distributions in the 7,500S and 230S pellets. The percentages of the neutral lipids of the 35S pellet which were monoglycerides, diglycerides, and fatty acids were significantly greater than the corresponding percentages of the neutral lipids in the 7,500S and 230S pellets. The percentage which was triglyceride in the 35S pellet was significantly less than the corresponding percentages in the 7,500S and 230S pellets. The percentages of the neutral lipids of the 230S and 35S pellets which were cholesterol were nearly identical and were about twice the corresponding percentage in the 7,500S pellet.

In order that a comparison can be made with the data in Table 15, the data in the column in Table 3 marked "Percentage of membrane lipids (%)" has been recalculated on the basis that the total neutral lipids are 100%. The recalculations are presented in Table 31.

In Table 31 the percentage given for triglycerides is similar to the corresponding percentages given for the 7,500S and 230S pellets in Table 15. Also, the percentage given for carotenoids, squalene, and cholesterol esters is similar to the percentages given for the "leading band" of the 7,500S and 230S pellets.

An overall comparison of the data in Table 15 and 31 shows that

the neutral lipid preparation of Thompson et al. (1961) contained a larger percentage of diglycerides and a smaller percentage of fatty acids than did the neutral lipids isolated in the research reported here. The fact that the 35S pellet neutral lipids contained 17% diglycerides may seem to contradict the previous statement; it does not, however, since only 7.1% (cf. Table 12) of the sum of lipids of the three pellets resided in the 35S pellet.

Table 29 gives the averages of data presented in Tables 23-28. Each mole of lysolecithin, sphingomyelin, lecithin, or cephalin contains one mole of phosphorus. Therefore, a figure in Table 29 represents a mole percentage of the total number of moles of phospholipid in each pellet.

The distributions of the major classes of phospholipids in the 7,500S and 230S pellets are virtually identical. The same distribution in the 35S pellet is different from those of the 7,500S and 230S pellets. The distribution in the 35S pellet includes a significantly higher percentage of sphingomyelin and a significantly lower percentage of lecithin than in the 7,500S and 230S pellets. The percentage cephalin is virtually the same for all three pellets.

The data in Table 29 can be compared with the distribution of the major classes of phospholipids in whole milk given in Table 2. Comparison shows that the phospholipids of whole milk contain more cephalin and less lecithin on a percentage basis than do the phospholipids of the isolated membrane fraction.

It has been shown above that there is variation among the three pellets in the distribution of the major classes of phospholipids in each pellet. Also, it was seen that there was variation between

whole milk and the fat globule membrane fraction in the distribution of the major classes of phospholipids. These findings are clearly in disagreement with the claim of Patton et al. (1964) that little or no variation in the distribution of the major classes of phospholipids exists among various milk fractions.

Thus far, it has been seen that the neutral lipids of the 35S pellet contain greater percentages of monoglycerides, diglycerides, and fatty acids than do the neutral lipids of the other two pellets. Also, the polar lipids of the 35S pellet contain a greater percentage of sphingomyelin than do the polar lipids of the other two pellets. It can be noted that monoglycerides, diglycerides, fatty acids, and sphingomyelin all have two things in common. First, they are either straight- or branched-chain molecules. Second, they have protons which are capable of engaging in hydrogen bonding.

Table 31 Data from Table 3 recalculated on the basis that the neutral lipids are 100%

Lipid	Per cent of neutral lipids (%)
Monoglycerides	5.85
Diglycerides	10.20
Cholesterol	6.49
Fatty acids	7.92
Triglycerides	67.19
Carotenoids, squalene, and cholesterol esters	2.33

CONCLUSIONS

1. The total lipids of the 230S and 35S pellets contained higher percentages of polar lipids than did the total lipids of the 7,500S pellet.
2. The neutral lipids of the 230S and 35S pellets contained higher percentages of cholesterol than did the neutral lipids of the 7,500S pellet.
3. The neutral lipids of the 35S pellet contained higher percentages of monoglycerides, diglycerides, and fatty acids and a lower percentage of triglycerides than did the neutral lipids of the 7,500S and 230S pellets.
4. The polar lipids of the 35S pellet contained a higher percentage of sphingomyelin and a lower percentage of lecithin than did the polar lipids of the 7,500S and 230S pellets.
5. The phospholipids of whole milk contain more cephalin and less lecithin on a percentage basis than do the phospholipids of the isolated membrane fraction (the three pellets).

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