

COMPOSITION OF CELLULAR MEMBRANES IN THE PANCREAS OF THE GUINEA PIG

II. Lipids

J. MELDOLESI, J. D. JAMIESON, and G. E. PALADE

With the technical assistance of LOUISE M. EVANS

From The Rockefeller University, New York 10021. Dr. Meldolesi's present address is the Università degli Studi, Istituto di Farmacologia e di Terapia, Milan, Italy

ABSTRACT

The lipid composition of rough and smooth microsomal membranes, zymogen granule membranes, and a plasmalemmal fraction from the guinea pig pancreatic exocrine cell has been determined. As a group, membranes of the smooth variety (i.e., smooth microsomes, zymogen granule membranes, and the plasmalemma) were similar in their content of phospholipids, cholesterol and neutral lipids, and in the ratio of total lipids to membrane proteins. In contrast, rough microsomal membranes contained much less sphingomyelin and cholesterol and possessed a smaller lipid/protein ratio. All membrane fractions were unusually high in their content of lysolecithin (up to $\sim 20\%$ of the total phospholipids) and of neutral lipids, especially fatty acids. The lysolecithin content was shown to be due to the hydrolysis of membrane lecithin by pancreatic lipase; the fatty acids, liberated by the action of lipase on endogenous triglyceride stores, are apparently scavenged by the membranes from the suspending media. Similar artifactually high levels of lysolecithin and fatty acids were noted in hepatic microsomes incubated with pancreatic postmicrosomal supernatant. E 600, an inhibitor of lipase, largely prevented the appearance of lysolecithin and fatty acids in pancreatic microsomes and in liver microsomes treated with pancreatic supernatant.

In the first article of this series, we have described the separation of membrane subfractions from rough microsome-, smooth microsome-, and zymogen granule fractions isolated from homogenates of guinea pig pancreas. We have also shown that a cell membrane fraction can be obtained from the same source (1).

These fractions represent membrane-bounded compartments which are involved in the synthesis, segregation, intracellular transport, and discharge of secretory proteins. In an attempt to understand how the membranes of these compartments interact with one another during the secretory process, we have investigated their chemical composition and enzyme activities. In this paper the lipid composition of the membranes is described.

At the onset of this study, we anticipated that a small fraction of the digestive enzymes stored in the gland, particularly in zymogen granules and in the duct system, could be released during tissue homogenization and fractionation and thereupon cause artifactual degradation and alteration of membrane components. In this respect, it has been reported that pancreatic lipase is active in the cold (2) and that at least some proteases may become activated during cell fractionation (3). Indeed, our studies showed that released lipolytic enzymes extensively affect the lipid composition of the isolated membranes. These alterations have been evaluated and the data on membrane lipids have been corrected accordingly.

METHODS AND MATERIALS

General Procedures

Data on animals used, and procedures applied for cell fractionation, protein determination, and electron microscopy have been given in the preceding paper (1). Guinea pig liver microsomes were isolated by differential centrifugation (4).

Lipid Extraction

The lipids of homogenates and resuspended cell fractions were extracted for convenience overnight at 4°C under nitrogen with 20 volumes of 2:1 chloroform-methanol (v/v) and purified according to Folch et al. (5). The solvent was evaporated in a stream of nitrogen; the residue was redissolved in either chloroform or 1:1 chloroform-methanol, and subjected to thin layer chromatography.

Thin Layer Chromatography

Unless otherwise indicated, thin layer chromatographic plates were prepared according to Skipski et al. (6), using 0.5 mm layers of silica gel, DO type without binder (Camag, Muttenz, Switzerland), spread with a Desaga apparatus (Brinkmann Instruments, Westbury, N. Y.). They were prewashed overnight with 2:1 chloroform-methanol and activated at 110°C for 45 min prior to use.

Phospholipids were separated with chloroform-methanol-acetic acid-water (25:15:4:2, v/v) (6) and neutral lipids by the quantitative two-step procedure described by Skipski et al. (7) in which the plates were eluted with diisobutyl ether-acetic acid (96:4, v/v) followed by petroleum ether-diethyl ether-acetic acid (90:10:1, v/v).

To separate cerebrosides we used two different methods. The best results were obtained with a three-step development procedure on 75% D-O silica gel-25% Mg silicate (M. Woelm, Eschwege, Germany) alkaline plates (8). This development requires the following solvent mixtures in sequence: (a) acetone-pyridine-chloroform-water (40:60:5:4, v/v); (b) diethyl ether-pyridine-ethanol-2 M ammonia (65:30:8:2, v/v); and finally, (c) diethyl ether-acetic acid (100:3, v/v). The method permits the separation of glycolipids without prior removal of the other lipids. The second method involves mild alkaline hydrolysis of the lipid extract, followed by dialysis and lyophilization (9). The redissolved lipids are then applied to silica gel plates and the chromatogram is developed first with diethyl ether-acetic acid (100:0.5, v/v), to remove neutral lipids, and then with chloroform-methanol-water (60:35:4.7, v/v).

For separating gangliosides, both phases obtained by the Folch partition of the lipid extracts were sub-

mitted to mild alkaline hydrolysis, dialyzed, and lyophilized; the redissolved lipids were separated by means of two successive runs of the plates in the system described by Ledeen et al. (9) (chloroform-methanol-2.5 M ammonia, 60:40:9, v/v).

Separated lipids were detected on plates by exposure to iodine vapors (10) or by spraying with either rhodamine 6G (0.05% in ethanol), ninhydrin (0.1% in isopropanol), 40% sulfuric acid (11), orcinol (0.2% in 75% H₂SO₄) (12), or resorcinol (4% in 30% HCl containing 0.25 mM CuSO₄) (13). They were identified with the help of reference standards, as well as by their staining reactions.

For quantitative analysis, the separated phospholipid and neutral lipid spots were scraped off the plates, recovered using a Hirsch funnel (14), and extracted from the silica by means of the solvent mixtures recommended by Skipski et al. (6, 7). After evaporation of the eluant under nitrogen, the samples were analyzed for lipid phosphorus (15), and for neutral lipids (16). Recovery for neutral lipids and phospholipids was between 75–100% based on the loads applied to the chromatograms.

Cholesterol Determination

After evaporation of the lipid extract in a stream of nitrogen, the residue was dissolved in 7 ml of 2.4% KOH in ethanol-benzene-water (65:27.5:7.5, v/v), heated at 60°C for 2 hr and then thoroughly mixed with 2 ml of water and 8 ml of petroleum ether. The mixture was allowed to separate into its two phases; the upper one was collected and the surface of the lower one rinsed twice with petroleum ether. Upper phase and rinses were combined, the solvents evaporated, and total cholesterol assayed in the residue by gas-liquid chromatography. Gas-liquid chromatographic analyses were performed on an F and M model 400 gas chromatograph equipped with a flame ionization detector. The 6 ft column was packed with 1% DC 560 on gas chrom Q. Determinations were carried out at 242°C. Carrier gas was nitrogen at pressure of 20 psi. Peak areas were determined using a digital integrator model CRA 100 (Infotronics, Inc., Houston, Texas). To each sample, 70 µg of 5 α cholestane was added as standard. Free and esterified cholesterol were likewise assayed after prior separation by thin layer chromatography.¹

Enzyme Assays

The lipase assay contained 10 µmoles Tris-HCl buffer, pH 9, 50 µmoles NaCl, 6 µmoles CaCl₂, 0.75 µmoles Na deoxycholate, 0.05 ml enzyme, and 0.05 ml

¹ The authors are grateful to Dr. N. Spritz, Manhattan Veterans Administration Hospital, New York, for these analyses.

substrate in a total volume of 0.5 ml. The substrate consisted of 4% olive oil and 0.03% tripalmitin-¹⁴C carboxyl in 30.6 mM Na-deoxycholic acid (DOC)² emulsified in a Waring blender (17). After 3 min of incubation at 40°C, the reaction was stopped by adding 10 ml of 2:1 chloroform-methanol (v/v). The lipids were extracted, purified, and the neutral lipids separated by thin layer chromatography, as described above (7). Plates were sprayed with rhodamine 6G, and free fatty acid (FFA) spots scraped off the plates and transferred to liquid scintillation vials, containing Bray's phosphor (18). Radioactivity was measured using a liquid scintillation spectrometer (Mark I, Nuclear-Chicago Corporation, Des Plaines, Ill.).

Phospholipase A was assayed in the reaction mixture of Haas et al. (19), in 0.5 ml volume. After 5 and 10 min of incubation at 37°C the reaction was stopped by adding 10 ml of 2:1 chloroform-methanol; lipids were extracted and purified, and phospholipids separated by thin layer chromatography as described (6). Lipid spots were detected by exposure to iodine vapors. Lysolecithin was scraped off the plate, and its lipid phosphorus was measured as described above (15).

Materials

The materials were obtained from the sources indicated below.

SOLVENTS (ALL REAGENT GRADE): chloroform, methanol, petroleum ether, and ammonium hydroxide: Merck Chemical Division, Merck and Co., Inc., Rahway, N. J.; pyridine, diethyl ether, and acetic acid: Mallinckrodt Chemical Works, St. Louis, Mo.; diisobutyl ether: Eastman Chemical Products, Inc., Rochester, N. Y.

REFERENCE AND RADIOACTIVE LIPIDS: tripalmitin: Sigma Chemical Co., St. Louis, Mo.; lecithin, lysolecithin, phosphatidyl serine, cholesterol, palmitic acid, and phosphatidyl ethanolamine: Mann Research Labs Inc., New York; sphingomyelin and phosphatidic acid (Ca salt): General Biochemicals, Chagrin Falls, Ohio; ceramide monohexoside, lysophosphatidyl ethanolamine, phosphatidyl inositol, and lecithin-¹⁴C carboxyl (SA, 0.27 mCi/mmole): Applied Science, State College, Pa.; ceramide dihexoside (cytolipin-H): Miles Yeda, Ltd., Rehovoth, Israel; tripalmitin-¹⁴C carboxyl (SA, 3.2 mCi/

² The following abbreviations are used in this paper: BSA, bovine serum albumin; DFP, diisopropyl-fluorophosphate; DOC, deoxycholic acid; E 600, diethyl-p-nitrophenyl phosphate; EDTA, ethylenediaminetetraacetate; FFA, free fatty acids; PCMB, p-chloromercuric benzoate; PMS, postmicrosomal supernate; PLP, phospholipids.

mmole): Tracerlab, Waltham, Mass. Purified beef brain gangliosides and Tay-Sachs gangliosides were a generous gift of Dr. R. Ledeen, Albert Einstein College of Medicine, New York; palmitoyl cholesterol was a gift of Dr. Jules Hirsch, The Rockefeller University, New York.

ENZYME INHIBITORS: p-chloromercuric benzoate (PCMB), Sigma Chemical Co.; diisopropylfluorophosphate (DFP): Mann Research Labs Inc. diethyl-p-nitrophenylphosphate (E 600): K & K Laboratories Inc., Plainview, N. Y.

DYES: rhodamine-6-G: Matheson, Coleman and Bell, Norwood, Ohio; resorcinol: Eastman Chemical Products, Inc.; orcinol: Sigma Chemical Co. ninhydrin: E. Merck Ag, Darmstadt, Germany.

RESULTS

Lipid Composition of Pancreatic Membranes

The phospholipid composition of pancreatic rough and smooth microsomal membranes, zymogen granule membrane, and plasma membrane is summarized in Table I.³ In all fractions, phospholipids account for only 30-37% of the total lipids. Lecithin and phosphatidyl ethanolamine are the major phosphatides comprising 50-60% of the total. Lecithin is more concentrated in rough microsomal membranes, whereas phosphatidyl ethanolamine is about equally distributed among all fractions, as are the minor phospholipids, phosphatidyl inositol and phosphatidyl serine. Of all the phospholipids, the distribution of sphingomyelin is the most interesting. This lipid is a minor component of the rough microsomal membrane but its concentration sharply increases in the smooth microsomal membrane and, especially, in the zymogen granule membrane and the plasma membrane.

Very little lipid phosphorus is found at the chromatogram front to which acidic phospholipids, such as cardiolipin and phosphatidic acid, are known to move. Since a high concentration of cardiolipin has been found in mitochondria of all tissues so far investigated (20, 21), this observation is consistent with the low level of enzymatically measurable mitochondrial contamination in our fractions (1).

All fractions have a surprisingly high content of

³ All these preparations are cell fractions or derived subfractions which consist primarily, but not exclusively, of the membranes mentioned. Hence, it is for convenience only that the terms marked above are used here and in the rest of the text.

TABLE I
Phosphatide Composition of Cellular Membranes in the Guinea Pig Pancreas

	Rough microsomal membranes (4)	Smooth microsomal membranes (4)	Zymogen granule membranes (3)	Plasma membranes (3)
	%	%	%	%
Origin	3.3 <i>2.2-4.5</i>	1.7 <i>1.0-2.4</i>	3.1 <i>1.6-4.2</i>	4.1 <i>3.2-5.9</i>
Sphingomyelin	3.5 <i>2.3-4.9</i>	14.2 <i>11.9-14.7</i>	23.5 <i>22.0-26.0</i>	19.2 <i>17.6-20.0</i>
Lecithin	34.7 <i>30.0-40.0</i>	26.4 <i>22.6-30.0</i>	21.5 <i>16.0-26.0</i>	24.7 <i>21.2-29.4</i>
Lysolecithin	13.1 <i>8.6-22.0</i>	13.1 <i>6.2-20.6</i>	9.1 <i>6.1-13.4</i>	7.1 <i>3.4-13.4</i>
Ph-ethanolamine	35.8 <i>30.0-40.0</i>	36.5 <i>30.0-43.5</i>	31.5 <i>29.0-34.0</i>	34.4 <i>32.0-37.3</i>
Ph-Serine + Ph-inositol	5.6 <i>3.0-10.0</i>	4.8 <i>2.5-6.5</i>	5.0 <i>4.6-5.4</i>	4.7 <i>3.2-6.0</i>
Front	4.2 <i>2.6-5.0</i>	3.5 <i>2.0-5.5</i>	5.9 <i>5.3-7.2</i>	4.7 <i>2.8-6.1</i>
PLP/total lipids	0.35 <i>0.33-0.37</i>	0.32 <i>0.31-0.35</i>	0.32 <i>0.30-0.34</i>	0.33 <i>0.30-0.35</i>

Values given are averages.
Number of experiments is shown in parentheses.
Ranges are in italics.
Recoveries of PLP/g of tissue are given in the preceding paper.

TABLE II
Neutral Lipids of Cellular Membranes of the Guinea Pig Pancreas

	Rough microsomal membranes	Smooth microsomal membranes	Zymogen granule membranes	Plasma membranes
	%	%	%	%
Total cholesterol (4) (μ moles/ μ moles PLP)	0.12 <i>0.06-0.17</i>	0.47 <i>0.39-0.57</i>	0.55 <i>0.43-0.61</i>	0.51 <i>0.48-0.54</i>
Free cholesterol (%) (2)	65 <i>64-66</i>	86 <i>84-87</i>	74 <i>71-77</i>	73 <i>71-75</i>
Ester. cholesterol (%) (2)	35 <i>34-36</i>	14 <i>13-15</i>	26 <i>23-29</i>	27 <i>25-29</i>
FFA/total lipids (2) (wt/wt)	0.58 <i>0.56-0.60</i>	0.57 <i>0.55-0.59</i>	0.54 <i>0.53-0.55</i>	0.54 <i>0.51-0.57</i>

Values given are averages.
Ranges are in italics.
Number of experiments is shown in parentheses.

lysolecithin, which varies considerably from experiment to experiment. In addition to chemically measurable amounts of lysolecithin, two minor ninhydrin-positive spots, representing most likely lysophosphatidyl ethanolamine and a trace amount of lysophosphatidyl serine, are detected in all fractions. The former migrates between lecithin and phosphatidyl serine and partially overlaps the lecithin spot, while the latter is positioned between sphingomyelin and lecithin. These lyso derivatives are present in too small a concentration to be measurable chemically. The possible origin of lysophosphatides will be discussed later.

Table II shows the distribution of neutral lipids. All membranes contain a large amount of free fatty acids, accounting for the majority of the total lipids (≈ 51 – 60%). Cholesterol shows a distribution similar to that of sphingomyelin: low concentration in rough microsomal membranes, in

contrast with much higher concentrations in the other membrane fractions. A sizable percentage of the total cholesterol is esterified (15–35%). Other neutral lipids such as triglycerides and monoglycerides are present in small amounts in all membrane fractions. Diglycerides are usually absent. Traces of esters of fatty acids, most likely methyl esters, are often observed. They have been described previously in the pancreas of several species (22–24). In our fractions, they are apparently a preparation artifact (25), since they have been found after chloroform-methanol, but not after diethyl ether-acetone (1:3) extraction.

Isolated membranes contained glycolipids, but their quantitative determination was not possible because of the small yield of our fractions. With both thin layer chromatographic systems used for cerebroside, we were able to identify several discrete spots that stained violet upon spraying

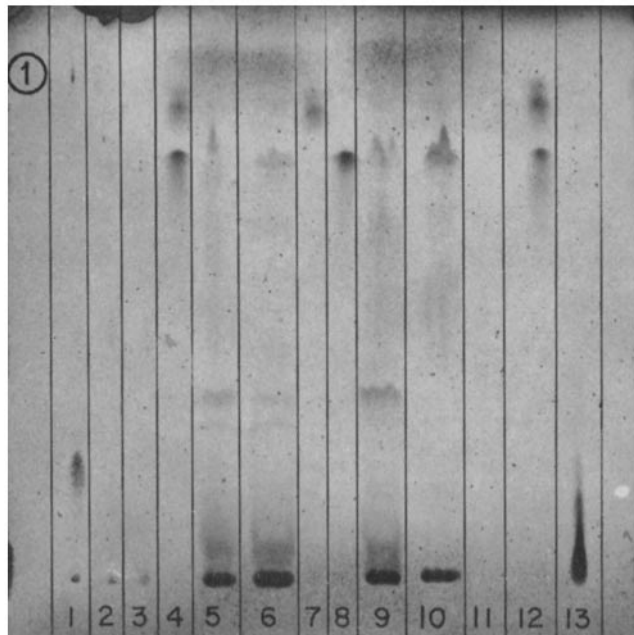


FIGURE 1 Thin layer chromatography of cerebroside. Preparation and development of plates as described by Skipski et al. (1967). Lane 1, Tay-Sachs gangliosides ($\sim 10 \mu\text{g}$). Lane 2, phosphatidic acid ($\sim 5 \mu\text{g}$). Lane 3, mixture of phospholipid standards ($\sim 5 \mu\text{g}$). Lane 4, ceramide-mono-hexoside standard ($\sim 20 \mu\text{g}$), upper spots; and ceramide-dihexoside standard ($20 \mu\text{g}$), lower spot. Lane 5, lipid extract of rough microsomes containing $\sim 0.5 \mu\text{mole}$ of lipid P. Lane 6, lipid extract of smooth microsomes, containing $\sim 0.5 \mu\text{mole}$ of lipid P. Lane 7, ceramide-mono-hexoside standard ($\sim 20 \mu\text{g}$). Lane 8, ceramide-dihexoside ($20 \mu\text{g}$). Lane 9, lipid extract from zymogen granule membranes, containing $\sim 0.5 \mu\text{mole}$ of lipid P. Lane 10, lipid extract of plasma membranes, containing $\sim 0.3 \mu\text{mole}$ of lipid P. Lane 11, mixture of neutral lipid standards ($\sim 10 \mu\text{g}$). Lane 12, standards as in lane 4. Lane 13, beef brain gangliosides ($\sim 20 \mu\text{g}$). Detection, 40% sulfuric acid. Note that the neutral lipids (lane 11) have moved off the front of the chromatogram and are not visible.

with orcinol, a reaction typical of sugars. In Skipski's system (8), in which only cerebrosides are separated, the major component had the same R_f as ceramide dihexoside and appeared to be concentrated especially in the plasma membrane although it was also present in other fractions (Fig. 1). A trace of ceramide monohexoside was present in all the fractions, as well as traces of slower moving glycolipids. An even slower moving glycolipid is present in rough and smooth microsomal membranes and zymogen granule membranes, but not in plasma membranes. In the second chromatographic system used, in which lipids are submitted to mild saponification prior to chromatography, this slow-moving component overlaps sphingomyelin which, of the phospholipids, is alkali resistant. Gangliosides were not detectable in any membrane fraction.

As we have already anticipated, the results suggest that the lipid pattern of our membrane fractions is extensively altered during preparation. Thus, we have always found in these membranes large amounts of lysolecithin (in some experiments, as high as 22% of the total phospholipids), and have detected the presence of other lysophospholipids, such as lysophosphatidyl ethanolamine and lysophosphatidyl serine, while in other tissues lysophosphatides are known to represent minor or undetectable components. To our knowledge, high concentrations of lysolecithin (of the order of 15%) have been observed previously only in secretory granules isolated from the adrenal medulla of several species (26, 27). Furthermore, free fatty acids are present in large amounts in pancreatic membranes, whereas they have been found only in trace amounts in cell fractions isolated from other tissues. These unusual findings could result from endogenous lipolytic activity during the homogenization and fractionation of the pancreatic tissue.

Experiments with Radioactive Lipids

To check this assumption, pancreatic homogenates were incubated at 4°C for 30 min with lecithin-¹⁴C carboxyl or tripalmitin-¹⁴C carboxyl and then processed to obtain microsomal membranes and zymogen granule membranes. Lipids, extracted from both total homogenates and isolated fractions were separated by thin layer chromatography and counted. The results are shown in Tables III and IV. Both tripalmitin and, especially, lecithin are actively hydrolyzed by the

TABLE III
Hydrolysis of Exogenous Lecithin-¹⁴C by Pancreatic Homogenates and Trapping of Released Fatty Acids by Microsome and Zymogen Granule Membranes

	Distribution of label		
	Lecithin	Lyso- lecithin	Front (FA)
	%	%	%
Homogenate before incubation	69.3	1.5	29.2
Homogenate after incubation	5.3	22	72.7
Total microsomal membranes	1.7	5.3	93
Zymogen granule membranes	1.6	1.3	97.1

20 ml of pancreatic homogenate was mixed at 4°C with a suspension of lecithin-¹⁴C carboxyl (2.2 μ Ci in 0.3 M sucrose). Lipids from 0.5 ml of the mixture were immediately extracted (homogenate before incubation). The rest was incubated for 30 min at 4°C; at the end of the incubation, lipids from a 0.5 ml sample were extracted (homogenate after incubation), while the rest of the mixture was fractionated as described. The isolated fractions were resuspended in 50 mM NaCl-140 mM NaHCO₃, pH 7.8, and recentrifuged to recover their membranes. From all preparations, lipids were extracted with 20 vol chloroform-methanol, 2:1, and the extracts were purified and chromatographed as given under Methods. Lecithin-¹⁴C carboxyl (labeled in both fatty acids) was suspended in 0.3 M sucrose by sonication (5 \times 20 sec at setting 8 amp, with cooling, by a fine tip Branson sonifier [Branson Instrument Inc., Stamford, Conn.]). Recovery of applied label ranged from 69% to 88%.

homogenate. In the case of lecithin, hydrolysis after 30 min is extensive, 72% of the radioactivity appearing at the front of the chromatogram (where neutral lipids, including free fatty acids migrate) and 22% in the lysolecithin spot. Moreover, the results show that membrane fractions isolated from such homogenates bind exogenous radioactive lipids, most of which are free fatty acids.

From these results we conclude that: (a) non-structural⁴ phospholipids and neutral lipids (triglycerides) are efficiently hydrolyzed, even at 4°C, by pancreatic enzymes, and (b) the released fatty

⁴ In the sense of not integrated (membrane-bound) in subcellular structures.

TABLE IV
Hydrolysis of Exogenous Tripalmitin-¹⁴C by Pancreatic Homogenates and Trapping of Released Fatty Acids by
Microsome and Zymogen Granule Membranes

		Distribution of label			
		TG	FFA	DG	MG
		%	%	%	%
Experiment 1	Homogenate before incubation	86.4	8.2	4.9	0.5
	Homogenate after 30 min incubation	78.2	14.5	5.9	1.4
	Total microsomal membranes	37.7	50.0	5.9	6.4
	Zymogen granule membranes	24.1	66.6	7.7	1.7
Experiment 2	Homogenate before incubation	83.8	8.8	5.4	1.9
	Homogenate after 10 min incubation	75.9	17.1	4.8	2.2
	Homogenate after 30 min incubation	66.6	22.3	7.2	4.6
	Microsomal membranes	15.9	62.2	10.9	8.7

20 ml of pancreatic homogenate was mixed at 4°C with an emulsion of tripalmitin-¹⁴C carboxyl (5 μCi, labeled in all three of the fatty acids). A sample was immediately removed and its lipids extracted with 20 vol 2:1 chloroform-methanol (homogenate before incubation). Additional samples were incubated at 4°C for 10 and 30 min and their lipids extracted at the end of the incubation (homogenate after incubation). Fractions were isolated from the rest of the homogenate after 30 min of incubation at 4°C. They were resuspended in 50 mM NaCl-140 mM NaHCO₃, pH 7.8, and recentrifuged to recover their membranes. In Experiment 1, the emulsion of tripalmitin-¹⁴C was prepared as described in Table III, while in Experiment 2, it was prepared by suspending the label in 30 mM DOC by means of a Waring blender (full speed, for 6 min with cooling). Recoveries of applied label ranged from 76% to 99%.

acids are effectively bound to, or trapped within, pancreatic membranes in such a way that they are not removed by subsequent washings with NaCl-NaHCO₃ solutions. The pancreas is known to contain large amounts of nonstructural lipids, primarily triglycerides (28), in the form of lipid droplets within acinar cells, and especially within the adipose cells of the stroma. These lipid droplets can be the source of at least part of the free fatty acids recovered in isolated fractions.

Mixing Experiments with Liver Microsomes

Additional experiments were carried out to test whether structural lipids, i.e. lipids built into biological membranes, also are susceptible to the lipolytic activity of pancreatic enzymes under the usual conditions of cell fractionation. Since the data given so far suggest that membranes isolated from pancreatic homogenates are already altered

by lipolysis (hence, unsuitable for such experiments), we turned to the guinea pig liver, in particular to liver microsomes, as a source of unaltered membranes. Published data indicate that the lysolecithin and free fatty acid content of liver microsomes from other species is very low (20, 21, 29).

Liver microsomes (guinea pig) were resuspended in a postmicrosomal supernate (PMS) obtained from either the liver or the pancreas of the same animal. The suspensions were kept for 30 min at 4°C, after which the microsomes were recovered by centrifugation and their membranes isolated as indicated in Table V. The results obtained in four such mixing experiments varied quantitatively but showed a consistent trend. Data from two experiments in which the most marked changes were recorded showed that upon NaCl-NaHCO₃ extraction, nearly 80% of the proteins, 40% of the

TABLE V
Neutral Lipid Composition of Hepatic Microsomal Membranes after Incubation in Hepatic or Pancreatic Postmicrosomal Supernate

	Hepatic microsomes + hepatic PSM		Hepatic microsomes + pancreatic PSM	
	Total lipids	Tissue wet weight	Total lipids	Tissue wet weight
	%	mg/g	%	mg/g
Triglycerides	12.6	1.20	6.5	0.52
Monoglycerides	2.1	0.20	22.1	1.75
Free fatty acids	4.4	0.42	46.4	3.62
Free cholesterol	4.1	0.39	3.53	0.28

Microsomes isolated from ~2 g of guinea pig liver were resuspended in 1 ml of 0.3 M sucrose and mixed with 9 ml of postmicrosomal supernate derived from 10% homogenate of either the liver or the pancreas of the same animal. The suspensions were incubated at 4°C for 30 min. The microsomes were recovered by centrifugation at 50,000 rpm for 60 min (Spinco No. 50 rotor) and subsequently extracted with 50 mM NaCl-140 mM NaHCO₃, pH 7.8. Microsomal membranes were recovered from the extraction mixture by centrifugation for 60 min at 50,000 rpm in the same rotor.

phospholipids, and nearly all of the RNA of liver microsomes were no longer sedimentable after incubation with pancreatic PMS. The corresponding microsomal membranes contained three times more neutral lipids than the controls; monoglycerides were greatly increased, and free fatty acids accounted for ~46% of the total lipids. In controls, fatty acids were present only in small concentrations (Table V).

The over-all recovery of phospholipids in treated microsomes was low (~32% of the controls). Moreover, the losses were not distributed equally among phospholipid classes: lecithin, phosphatidyl ethanolamine, and sphingomyelin recoveries being 11%, 23%, and 65%, respectively, of the control values. As a result, the phospholipid distribution was altered (Table VI): lecithin was greatly decreased, with a concomitant increase in lysolecithin, which became the predominant phospholipid; a moderate decrease in the concentration of phosphatidyl ethanolamine was evident and phosphatidyl serine and phosphatidyl inositol were no longer detectable; by contrast, the relative concentration of sphingomyelin in-

TABLE VI
Phosphatide Composition of Hepatic Microsomal Membranes after Incubation with either Hepatic or Pancreatic Postmicrosomal Supernatant

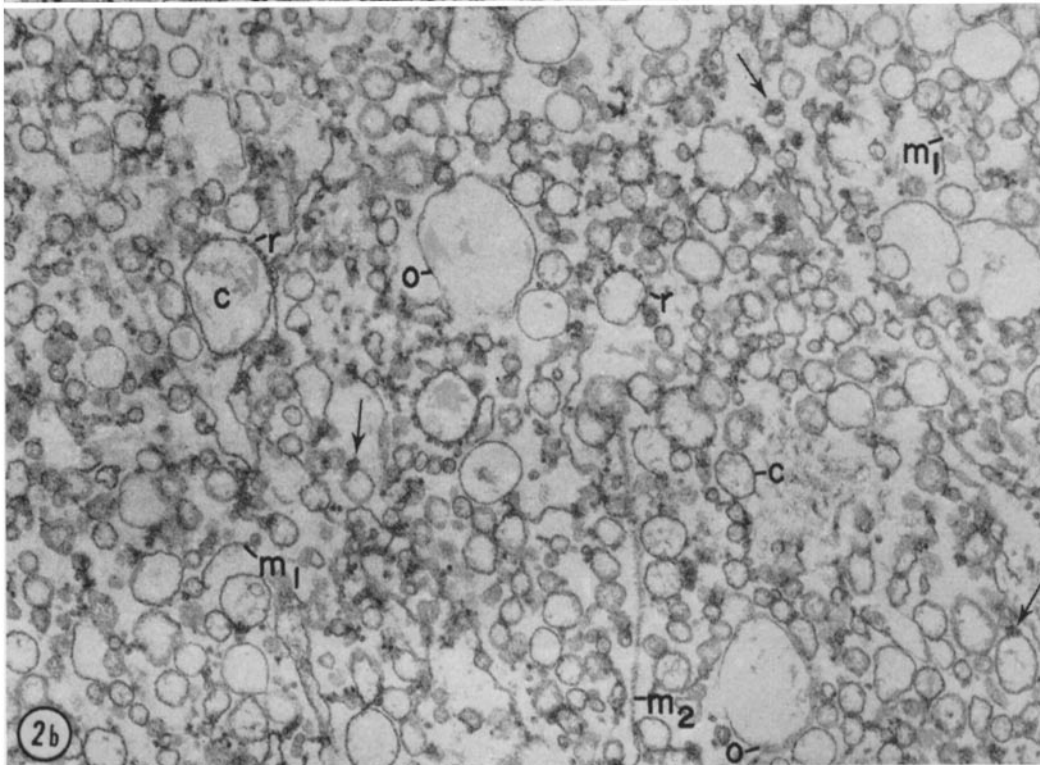
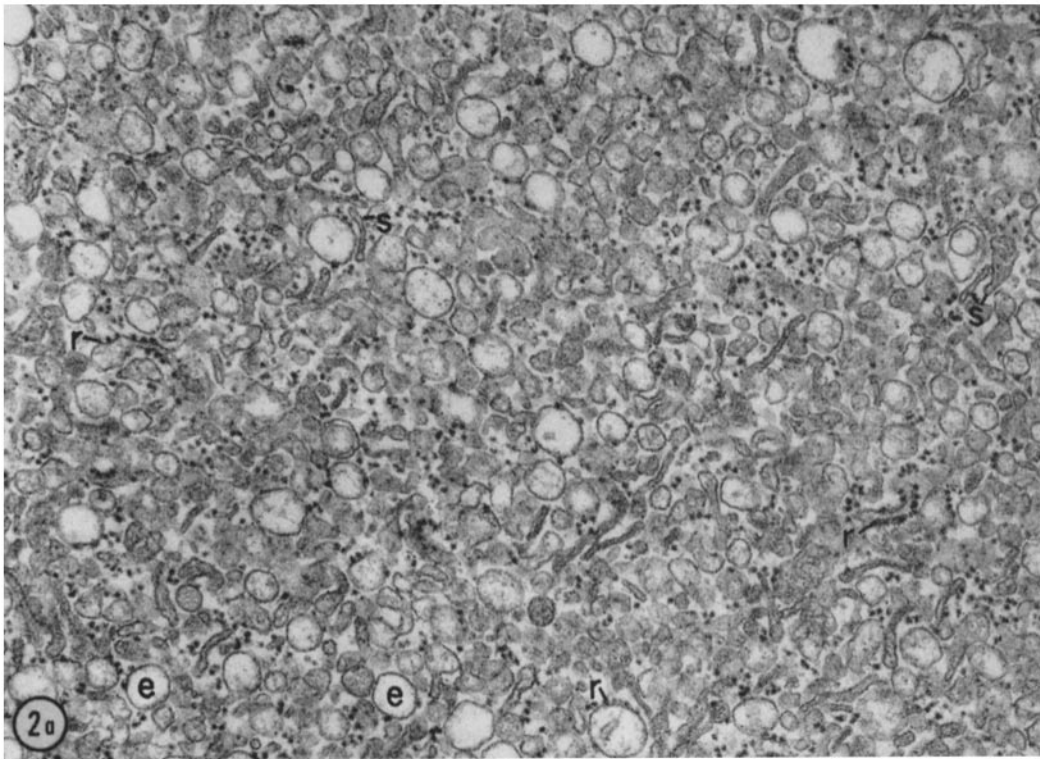
	Hepatic microsomes + hepatic PMS	Hepatic microsomes + pancreatic PMS
	Total PLP	Total PLP
	%	%
Origin	0.7	2.0
Sphingomyelin	2.8	5.7
Lecithin	51.1	17.8
Lysolecithin	0.6	27.5
Ph-ethanolamine	19.9	14.5
Ph-serine + Ph-inositol	12.0	0
Unknown phospholipid	0	19.5
Front	12.9	13.0
% of recovery of phospholipids applied to the plate	88.5	88.0

Experimental conditions as given in Table V. Values are the average of two highly consistent experiments.

creased twofold. In addition, a new phospholipid which accounted for nearly 20% of the recovered phosphatides was detected: it was ninhydrin-negative and migrated between phosphatidyl serine and phosphatidyl ethanolamine.

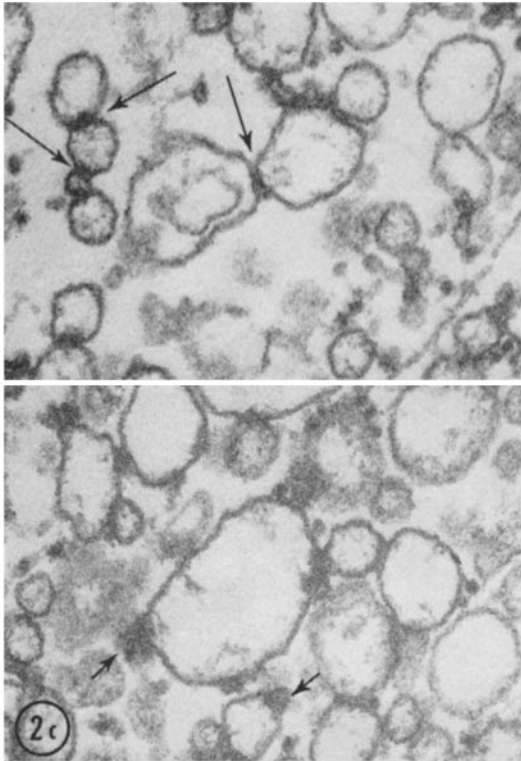
Two mechanisms could explain the net decrease of phospholipids in treated microsomes: (a) fragmentation of microsomal vesicles to small membrane pieces, no longer sedimentable under the usual conditions of isolation; and (b) degradation of phospholipids, especially lecithin, to fatty acids and water-soluble glycerophosphoryl compounds. The first mechanism is consistent with the relatively large amounts of phospholipids which remain in the bicarbonate extraction fluid after the recovery of treated microsomes, while the second could explain the low recovery of phospholipid phosphorus. In fact, the latter mechanism appears to be more important, since the recovery of lipolysis-insensitive compounds, e.g. sphingomyelin and cholesterol, is much better (~70%) than that of other phospholipids.

The fatty acids accumulated in microsomal membranes cannot be accounted for by hydrolysis of structural phospholipids only, for their amount is in molar excess with respect to degraded phosphatides; hence, some of them must come from



other sources, such as nonstructural lipids,⁵ as indicated by our experiments with radioactive triglycerides.

⁵ This conclusion is also supported by the observation that in mixing experiments in which the effects of the pancreatic PMS on the phospholipids of liver microsomes were less pronounced the accumulation of free fatty acids in microsomal membranes was still very large.



The morphology of liver microsomes incubated in pancreatic supernate is shown in Fig. 2. Most microsomal vesicles appear swollen but retain a continuous limiting membrane; a few are ruptured. All have lost either in part or completely their content and some appear to have lost their attached ribosomes (compare Fig. 2 *a* to 2 *b*). In addition, many vesicles show dense protrusions which are continuous with, or included within, their limiting membranes, and which, at higher magnifications and in favorable sections, appear to result from the local accumulation of electron-opaque material in between the two dense layers of the membrane (Fig. 2 *c*). Frequently the affected vesicles fuse with one another at the level of the protrusions (Fig. 2 *c*). Such protrusions are identical to those described in liver and muscle microsomes incubated with phospholipase C, and believed to be local accumulation of diglycerides resulting from the hydrolysis of structural phospholipids (30, 31). Since in our membranes diglycerides were present only in trace amounts, it seems probable that other neutral lipids, e.g. monoglycerides or free fatty acids, can cause such structural alterations. Similar intramembrane accumulations have been observed in pancreatic microsomes isolated under usual conditions (32). Besides recognizable microsomal vesicles, the recovered pellet contains pieces of membranes, sometimes of considerable length, whose central light layer is widened and occasionally exploded.

We conclude, therefore, that upon brief incubation at 4°C with a pancreatic postmicrosomal supernate, the lipid composition of isolated liver microsomes is changed: in many respects, it becomes similar to that of pancreatic membrane fractions. The finding strongly suggests that the lipid composition of the latter is altered by the

FIGURE 2 *a* Guinea pig hepatic microsomes incubated for 30 min at 4°C in 0.3 M sucrose. As usual with guinea pig microsomes, the proportion of rough vesicles (*r*) is low and that of smooth vesicles (*s*) is high in the total microsome population. Nearly all vesicles are intact and most of them retain a finely granular content of moderate and variable density. Empty vesicles are marked *e*. $\times 46,000$.

FIGURES 2 *b* and 2 *c* Guinea pig hepatic microsomes incubated for 30 min at 4°C in pancreatic postmicrosomal supernate. Fig. 2 *b*: The microsomal vesicles appear generally swollen. Most of them are still closed, but some of them are ruptured and open (*o*). Membrane fragments (*m*₁) and membrane sheets (*m*₂) of considerable length are seen among these vesicles. Practically all microsomes have lost their content; those which retain part of it are marked *c*. Attached ribosomes (*r*) are still present on some vesicles. The arrows point out dense protrusions of the membrane of some microsomal vesicles. $\times 43,000$.

FIGURE 2 *c* Microsomal vesicles treated as for Fig. 2 *b*. Short arrows mark the dense protrusions of the microsomal membranes and the long arrows point out areas of membrane fusion. $\times 95,000$.

activity of endogenous pancreatic lipolytic enzymes during tissue homogenization and cell fractionation.

Experiments with Enzyme Inhibitors

To check this hypothesis, we investigated the effect of several enzyme inhibitors on the lipolytic activity of guinea pig pancreatic homogenates. Our purpose was to identify the enzyme(s) responsible for the alterations of the lipid composition of membranes, as well as to isolate membrane fractions whose lipid composition was unaltered.

Several lipolytic enzymes, such as lipase, phospholipase A and C, and lysophospholipase, have been shown to be highly active in the pancreas of different species, and several inhibitors of these enzymes have been studied. Recently, de Haas et al. have provided convincing evidence that in porcine, and probably also in bovine and human pancreas, phospholipase A is present as a zymogen whose activation requires traces of trypsin and takes place upon storage of the tissue (33). The enzyme is heat-stable at acid pH's: it is unaffected by heating to 70°C for 3 min at pH 4 (19, 34, 35), and it is inhibited only by 42% after boiling for 15 min (35). It requires Ca⁺⁺, and is completely inhibited by ethylenediaminetetraacetate (EDTA) (19, 35); it is inhibited by Pb⁺⁺ and Zn⁺⁺ (the human enzyme also by Ag⁺), but not by PCMB and E 600, irrespective of the concentrations tested (19, 35). DFP prevents the activation of the pro-phospholipase by inhibiting trypsin (33).

Lipase, by contrast, is known to be heat-labile and to be inhibited rapidly and irreversibly by high concentrations of E 600 (36) and less effectively by PCMB and iodine (37). The enzyme requires Ca⁺⁺ only under certain experimental conditions (17, 37). Lysophospholipase is inhibited by DOC (35).

It should be emphasized that purified pancreatic lipase is capable of hydrolyzing the ester bond in position 1 in most phospholipids, although at a slower rate than in glycerides (38). For this reason, the concomitant hydrolysis of triglycerides and phospholipids observed in our experiments could be due to a single enzyme, lipase.

The inhibitors discussed so far were tested and the results on the phospholipase and lipase activity of guinea pig pancreatic homogenates are shown in Table VII. Even high concentrations of EDTA did not prevent the hydrolysis of phospholipids, whereas Zn⁺⁺, Ag⁺ and, especially, E 600 were

TABLE VII
Effect of Different Inhibitors on Phospholipase A and Lipase Activity of Guinea Pig Pancreatic Homogenates

Inhibitor	Ph-lipase A		Lipase	
	activity*	activity*	activity†	activity‡
	%§	%§	%§	%§
EDTA 10 mM	90			
50 mM	90			
Ca ⁺⁺ 1 mM	87			
5 mM	83			
Zn ⁺⁺ 1 mM	100	42		
5 mM	0	15	31	29
Ag ⁺ 1 mM	6	26		
5 mM	0	3	68	83
Pb ⁺⁺ 1 mM	100	100		
5 mM	83	90		
I 0.01 mM	85	93		
0.05 mM	106	97		
PCMB 1 mM			100	120
DFP 10 mM			100	100
E 600 18 mM			56	100
100 mM	6	0	0	5
Heat (70°C × 3 min, pH 5.6)			0	0
Aging (48 hr at 4°C)			100	100

Homogenate samples were assayed as indicated under Methods; Ca⁺⁺ and Zn⁺⁺ were used as chlorides; Ag⁺ and Pb⁺⁺ as nitrates; 0.1 M DFP in isopropanol was diluted with 0.3 M sucrose; E 600 was emulsified in 0.08% DOC—0.3 M sucrose by means of a Waring blender; PCMB was dissolved in 0.3 M sucrose by alkalization with Na bicarbonate.

* Inhibitors were added to the reaction mixture of the enzyme assay.

† Inhibitors were added to the 0.3 M sucrose used for the homogenization of the tissue.

‡ Per cent of activity in controls.

capable of inhibiting completely the degradation of both phosphatides and glycerides. Lead and iodine were ineffective. Activities against both substrates did not increase upon storage and were destroyed by heating at 70°C for 3 min at pH 5.5. When inhibitors were added to the sucrose used for tissue homogenization, Zn⁺⁺ and E 600 inhibited both enzyme activities, whereas DFP, at a concentration known to eliminate completely any tryptic activity, was ineffective.

These results suggest that a single enzyme is responsible for both lipase and phospholipase

activities, since both activities are influenced in parallel by all the inhibitors tested. This enzyme behaves more like lipase since it is thermolabile, fully active in the fresh homogenate (even in the presence of DFP), and completely inhibited by high concentrations of E 600. However, in our experiments with radioactive lipids, we have shown that lecithin is split much more efficiently than tripalmitin by pancreatic homogenates, although lipase would be expected to act more readily on tripalmitin. Since the activity of lipolytic enzymes depends so much on the physical state of the substrate, and since the preparation of good micellar suspensions is much easier with lecithin than with tripalmitin, reaction rates cannot be used as a reliable criterion for enzyme identification.

Having found that E 600 and Zn^{++} inhibit the two lipolytic activities, we tried to isolate membrane fractions from pancreatic tissue homogenized in the presence of each inhibitor. While high concentrations of Zn^{++} made fractionation impossible, we succeeded in isolating a total microsomal fraction from tissue homogenized in sucrose-E 600. The yield was smaller than in the usual procedures, but by comparison with usual microsomes, the "E 600 microsomes" contained much less free fatty acids (Table VIII); and their lysolecithin content was dramatically reduced concomitantly with a quantitative increase in the amount of lecithin (Table IX).⁶ Their sphingomyelin content was unchanged. The protein and RNA decrease and the increased phospholipid/protein ratio were probably due to detachment of ribosomes and extraction of microsomal content by the 0.08% DOC used to emulsify the inhibitor, rather than to the inhibitor itself.⁷

The morphology of pancreatic microsomes isolated in sucrose, sucrose-0.8% DOC, or sucrose-0.08% DOC-E 600 is shown in Figs. 3 *a*, *b*, and *c*,

⁶ Since E 600 is lipid soluble and contains phosphorus, its presence interferes with the determination of phospholipids. Therefore, phospholipid phosphorus assays were performed after thin layer chromatography of the extract with diethyl ether-acetic acid (100:3, v/v), which leaves phospholipids at the origin while the E 600 moves with the solvent front.
⁷ Microsomes, isolated in homogenates made in 0.08% DOC-0.3 M sucrose appear to lose their attached ribosomes and are devoid of content. The lipid composition is unchanged with respect to the fraction isolated by the usual procedure.

respectively. Microsomal vesicles isolated in the presence of E 600 appear dilated and empty. Their membranes are free of electron-opaque protrusions. Ribosomes, attached as well as free (in the upper third of the pellet), appear noticeably enlarged (≈ 350 - 400 A in diameter).

The effectiveness of E 600 in inhibiting lipolysis was further demonstrated in an experiment in which liver microsomes were incubated in the cold with pancreatic microsomal supernate derived from a homogenate prepared in 0.3 M sucrose-2.75% E 600. Under these conditions, the lipid composition remained completely unaltered (data not shown). By electron microscopy we observed that the microsomal membranes retained their structure (Figs. 4 *a*, *b*) and did not show the protrusions or intramembrane accumulations of electron-opaque material noted in the absence of the inhibitor.

Washing of Pancreatic Membranes with Albumin

To determine whether and to what extent the fatty acids trapped in pancreatic membranes remain removable, we washed the isolated membranes with bovine serum albumin (BSA), which is known to bind avidly free fatty acids.

Pancreatic microsomes were resuspended either in saline or saline containing 1% BSA defatted according to the Chen (39). The ratio of BSA to microsome protein in the mixture was 6.7. After centrifugation at 50,000 rpm for 4 hr (Spinco 50 rotor) the supernates were removed, lyophilized, and dissolved in a small volume of water. Lipids from both pellets and supernatants were extracted and neutral lipids separated as described (Fig. 5). Compared to the pellet washed with saline, the BSA-washed pellet retained most of the cholesterol, cholesterol esters, triglycerides, diglycerides, and 79% of the phospholipids, but only 18.5% of the free fatty acids of the controls. The saline supernate contained only a trace of phospholipids, whereas the BSA supernate contained 21% of the phospholipids and 81.5% of free fatty acids. Other neutral lipids were undetectable. By repeating the washing, an additional amount of free fatty acids could be recovered in the BSA supernate.

DISCUSSION

Membranes prepared from pancreatic cell fractions (e.g., rough microsome-, smooth microsome-,

TABLE VIII
Chemical Composition of Pancreatic Microsomes Isolated by the Usual Procedure or in the Presence of 2.75% E 600

	Protein		Total lipids			Phospholipids			FFA		RNA	
	mg/g tissue wet wt	mg/g tissue wet wt	mg/g tissue wet wt	μg/mg protein	mg/g tissue wet wt	μg/mg protein	mg/g tissue wet wt	μg/mg protein	mg/g tissue wet wt	μg/mg protein	mg/g tissue wet wt	μg/mg protein
Pancreatic microsomes	21.5 (3)	6.5 (2)	309 (2)	2.4 (3)	111 (3)	3.7 (3)	171 (3)	5.5 (3)	253 (3)	5.0-5.8	235-265	
E 600 pancreatic microsomes	8.7 (3)	3.6 (2)*	431 (2)*	1.9 (3)*	214 (3)*	0.26 (3)	34 (3)	1.3 (3)	150 (3)	0.6-1.8	80-192	
	7.3-9.4	3.0-4.2	412-450	1.6-2.1	208-220	0.21-0.30	27-44	0.6-1.8	80-192			

* Values of total lipids and phospholipids in E 600 microsomes have been corrected for the presence of E 600 as described in footnote 6. Values given are averages; number of experiments is shown in parentheses; ranges are in italics.

TABLE IX
Phosphatide Composition of Pancreatic Microsomes Isolated by Usual Procedure or in the Presence of 2.75% E 600

	Pancreatic microsomes	E 600 pancreatic microsomes
	%	%
Origin	4.9 <i>4.7-5.0</i>	2.6 <i>2.1-3.4</i>
Sphingomyelin	7.4 <i>6.6-8.0</i>	7.4 <i>6.1-8.4</i>
Lecithin	25.9 <i>23.6-28.0</i>	43.6 <i>42.0-46.0</i>
Lysolecithin	22.1 <i>13.6-31.0</i>	4.5 <i>4.0-5.2</i>
Ph-ethanolamine	28.5 <i>21.5-33.0</i>	32.0 <i>20.0-36.0</i>
Ph-serine + Ph-inositol	6.1 <i>5.9-7.0</i>	4.8 <i>3.3-6.7</i>
Front	5.1 <i>3.7-5.9</i>	5.1 <i>3.7-6.0</i>
Recovery	96 <i>92-100</i>	97 <i>81-100</i>

Values of phospholipids in E 600 microsomes have been corrected for the presence of E 600 as described in footnote 6.

Values are the average of three experiments; ranges are in italics.

zymogen granule-, and plasmalemma fractions) have a content of free fatty acids and lysophosphatides (especially lysolecithin) much higher than that so far reported for any other type of membrane from any other tissue. The possible explanation of this unusual finding is degradation of membranes by pancreatic lipolytic and proteolytic enzymes with subsequent lipid redistribution. Some of these enzymes, e.g. lipase, are present in active form in the tissue and have measurable activity at 0°C (2). Others, e.g. phospholipase A and the proteases, occur as inactive precursors, but their activation during tissue homogenization and fractionation is not excluded (3).

Digestive enzymes and zymogens are known to be released partially and redistributed during cell fractionation and, as a result, appear in varied concentrations in the pancreatic PMS (3). The explanation we proposed above is supported by the finding that incubation of hepatic microsomes in pancreatic PMS at 4°C reproduces in the former the lipid distribution usually found in pancreatic microsomes. In addition, experiments carried out

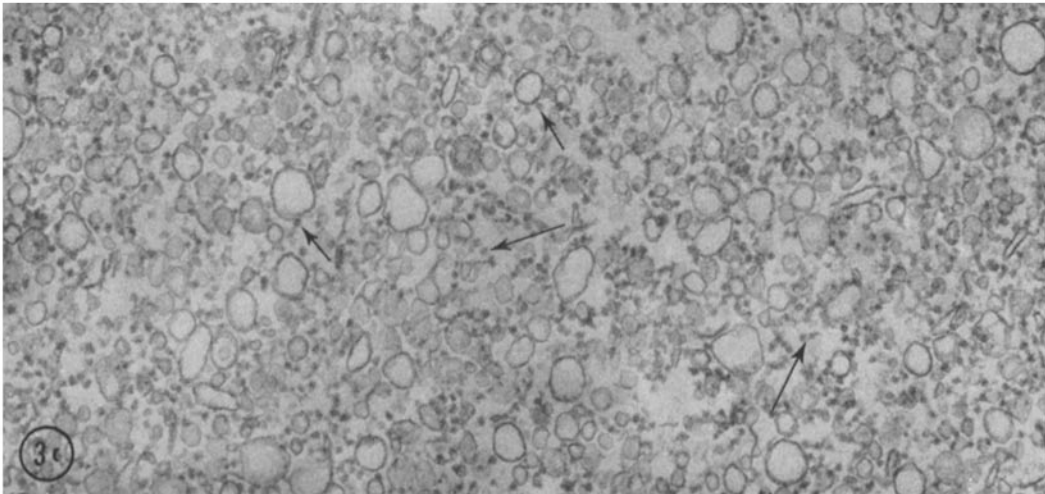
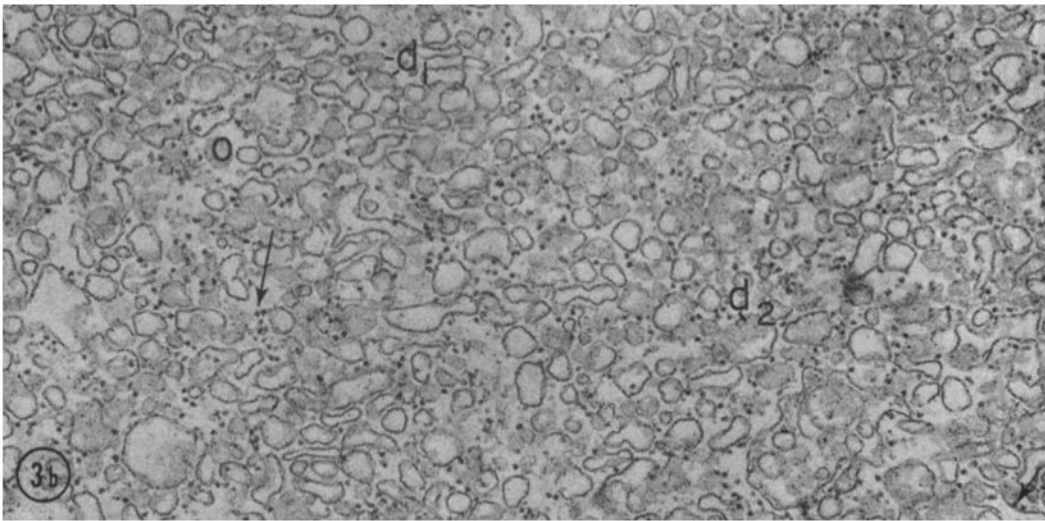
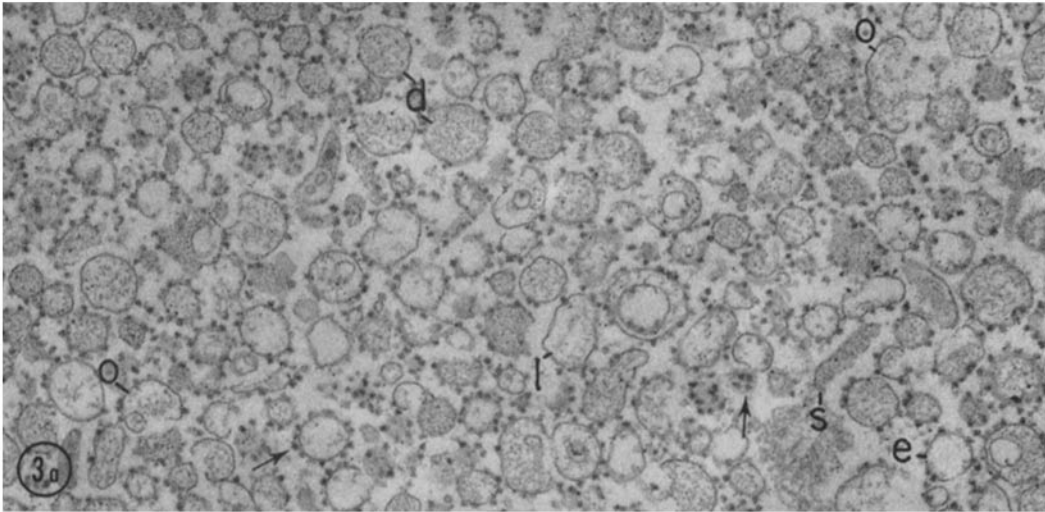
with a variety of inhibitors strongly suggest that the peculiarities noted in lipid distribution are accountable by lipase, rather than phospholipase activity. Indeed, pancreatic microsomes prepared in the presence of E 600, a powerful lipase inhibitor (36), have a low content of free fatty acids and lysophosphatides, whereas pancreatic microsomes prepared in the presence of DFP have the same lipid distribution as the controls. DFP inactivates trypsin and thereby is expected to prevent the activation of phospholipase A (33).⁸

Since we were not able to prepare the complete set of cell fractions from tissue homogenized in sucrose-E 600, we cannot ascertain that the lipids of all the fractions we studied are similarly affected, but we assume that what we have demonstrated for microsomes applies also for the other cell fractions and membranes derived therefrom.

On the basis of this assumption, we can conclude that the lysophosphatides found in our preparations are the result of deacylation of phosphatides preexisting *in vivo* in cellular membranes. The same does not apply, however, for free fatty acids, since they are present in a substantial excess over lysophosphatides. This excess most likely comes from other sources, particularly from the triglycerides of the tissue. This view is supported by the finding that labeled acyl groups of exogenous triglycerides introduced into a pancreatic homogenate are recovered in association with the membranes of derived microsomes. Under these conditions the membranes appear to function as effective traps for free fatty acids.

We do not know whether the trapped fatty acids are localized within the structure of the membranes or are adsorbed onto their surface. Some of the structural peculiarities of the microsomal membranes, i.e. the focal intramembrane accumulations of dense material, could be related to

⁸ In agreement with this conclusion we have observed that lysolecithin isolated from pancreatic microsomes contains a smaller percentage of saturated fatty acids by comparison to lecithin isolated from the same source (9.6% vs. 28.7%). Since in natural phospholipids saturated fatty acids are preferentially localized in position 1, it seems probable that our lysolecithin is mainly the two-acyl isomer. This could be produced through hydrolysis of lecithin by lipase (which is known to split the ester bond in position 2). The gas liquid chromatographic analysis of fatty acids (40) of microsomal phospholipids was kindly performed by Dr. C. Galli, Institute of Pharmacology, University of Milan, Milan, Italy.



this trapping, but the correlation cannot be extended to all fractions: similar accumulations are not found in the membranes of zymogen granules or in plasmalemmal fragments although they both have a high fatty acid content. Whatever their relationship to the membranes, the excess fatty acids can be removed extensively by washing with solutions of defatted albumin.

The results so far discussed support the assumption that excess free fatty acids represent extraneous compounds trapped in the membranes during the homogenization and fractionation of the tissue. If we correct our data in keeping with this assumption, the lipid distribution in pancreatic membranes becomes generally comparable to that found in membranes from other sources, although a series of peculiar features is retained as follows.

The corrected data (Table X) show that phospholipids are the major components of all pancreatic membrane lipids. They account for the largest percentage of the total lipids in rough microsomal membranes, in which the predominant phospholipids are lecithins. In this respect our findings are comparable to data available on microsomes prepared from liver (20, 21, 29, 41), adrenal cortex (41) and medulla (26), cardiac and skeletal muscle (42, 43), and several lines of cultured cells (44). Rough microsomal membranes are also characterized by low sphingomyelin content, a feature shared with microsomes isolated from a variety of sources (20, 21, 26, 29, 41-44) except kidney (41), the microsomal fraction of

which is expected to be heavily contaminated by plasmalemmal fragments. The cholesterol content of rough microsomal membranes is low, but appears to represent a genuine feature of this type of membrane rather than contamination by other cellular membranes. This interpretation is supported by the existence of different sphingomyelin/cholesterol ratios for each membrane fraction, and by the absence of enzymatic activities expected from plasma membrane or smooth microsome contaminants (45).

By comparison with their rough counterparts, the smooth microsomal membranes have a rather distinctive lipid composition characterized by a significant increase ($> 4 \times$) in sphingomyelin and cholesterol content and by a commensurate decrease in relative lecithin concentration. As already shown (46), this fraction represents primarily the light components of the Golgi complex and as such it cannot be compared to the usual smooth microsomal fraction isolated from liver homogenates. It is in part similar to Golgi-rich fractions isolated from rat (47, 48) and ox (49) liver, and to the "smooth II fraction" obtained by Dallner (29). The intracellular source of the latter fraction is unknown.

The zymogen granule membranes show a further increase in sphingomyelin content ($\approx 7 \times$) with a commensurate decrease in lecithin when compared to rough microsomal membranes. The cholesterol content increases slightly over that found in smooth microsomes and the over-all

FIGURE 3 *a* Guinea pig pancreatic microsomes isolated from a homogenate prepared in 0.3 M sucrose. Middle layer in the pellet. The fraction primarily consists of intact, spherical, and rough microsomal vesicles whose content varies in density from moderate (*d*) to light (*l*); some vesicles are nearly empty (*e*), and a few are ruptured (*o*). Smooth microsomes (*s*) are few in number. The arrows indicate attached ribosomes in either normal or grazing sections of the vesicles. At this level in the pellet, microsomes with dense protrusions of their limiting membrane (see Fig. 6 in reference 1) are rare; in this field there are none. $\times 38,000$.

FIGURE 3 *b* Guinea pig pancreatic microsomes isolated from a homogenate prepared in 0.3 M sucrose-0.08% DOC. Many microsomal vesicles have irregular profiles and most of them appear closed but empty; a few are ruptured (*o*). The moderate, even density of some vesicles is due to the complete (*d*₁) or partial (*d*₂) inclusion of their membranes in the thickness of the section. Few ribosomes are still attached (short arrows); most have been detached (long arrows) and appear scattered among the microsomal vesicles. $\times 38,000$.

FIGURE 3 *c* Guinea pig pancreatic microsomes isolated from a homogenate prepared in 0.3 M sucrose-0.08% DOC-2.5% E 600. The microsomal vesicles have relatively irregular profiles and, as in Fig. 3 *b*, are empty. Attached (short arrows) as well as free (long arrows) ribosomes appear noticeably enlarged. $\times 38,000$.

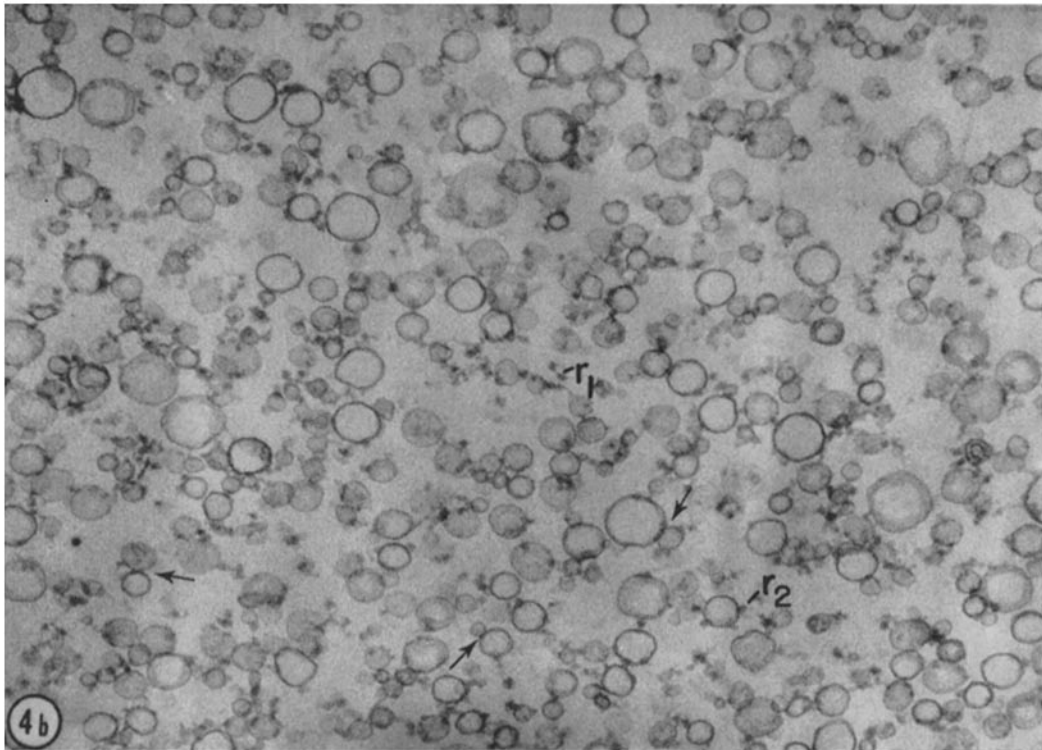
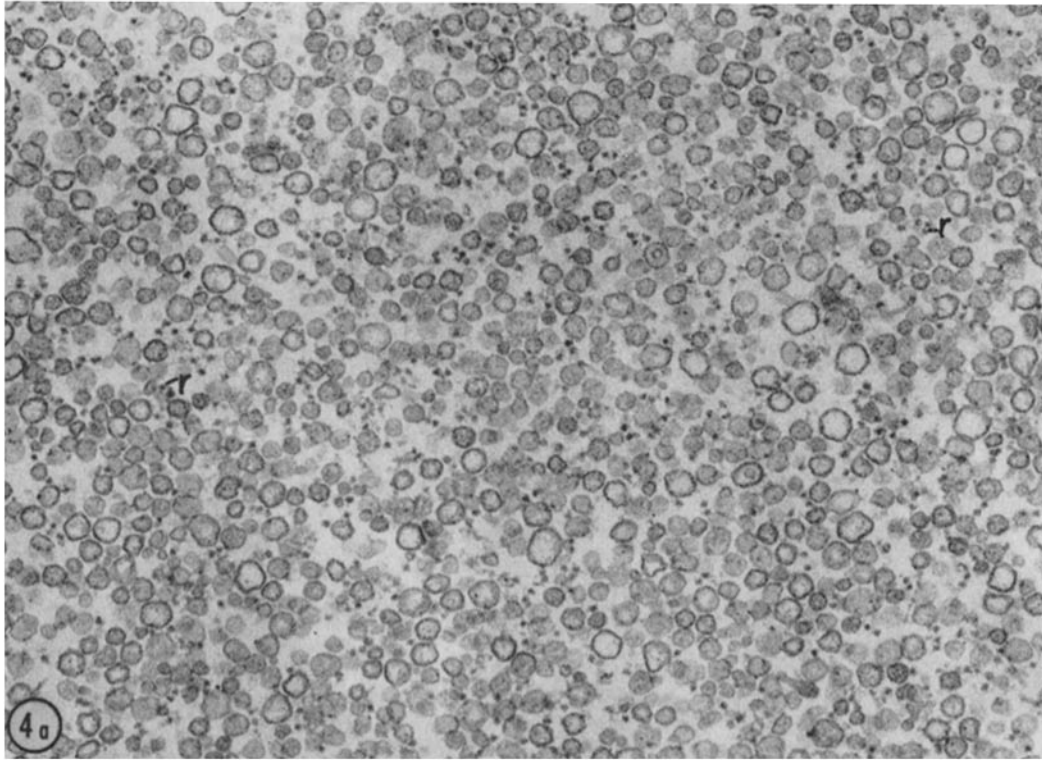


FIGURE 4 *a* Guinea pig hepatic microsomes incubated at 4°C with a PMS isolated from a pancreatic homogenate prepared in 0.3 M sucrose-0.08% DOC. In the presence of this small concentration of detergent, the microsomes appear as small, closed, rounded vesicles which have lost most of their content (compare with Fig. 2 *a*). Most ribosomes (*r*) are detached and appear scattered among the vesicles. $\times 40,000$.

FIGURE 4 *b* Guinea pig hepatic microsomes incubated at 4°C with a PMS isolated from a pancreatic homogenate prepared in 0.3 M sucrose-0.08% DOC-100 mM E 600. The microsomes prepared in the presence of E 600 are larger but otherwise generally similar to those prepared in sucrose-DOC (Fig. 4 *a*). There are few dense protrusions of their limiting membranes (arrows). Ribosomes free (*r*₁) as well as still attached (*r*₂) appear noticeably swollen (compare with Fig. 4 *a*). $\times 40,000$.

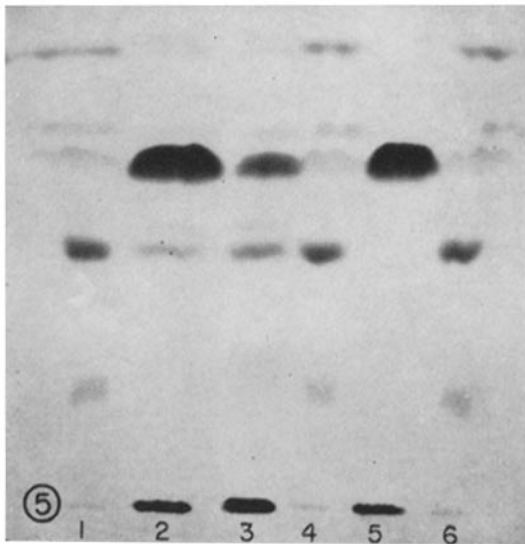


FIGURE 5 Thin layer chromatography of neutral lipids from pancreatic microsomes washed with saline or with saline containing 1% bovine serum albumin. Lane 1, lipid standards. From top to bottom: cholesterol palmitate ($\sim 30 \mu\text{g}$), tripalmitin ($\sim 50 \mu\text{g}$), palmitic acid ($\sim 50 \mu\text{g}$), cholesterol ($\sim 50 \mu\text{g}$), monopalmitin ($\sim 50 \mu\text{g}$). Lane 2, lipid extract from pancreatic microsomes, washed once with saline. It contains $\sim 500 \mu\text{moles}$ of lipid P. Lane 3, lipid extract from pancreatic microsomes washed once with saline containing 1% defatted BSA. It contains $\sim 800 \mu\text{moles}$ of lipid P. Lane 4, lipid standards as in lane 1. Lane 5, lipid extract of the saline-albumin washing solution of lane 3 microsomes. It contains $\sim 200 \mu\text{g}$ of lipid P. Lane 6, lipid standards as in lane 1. Detection, iodine vapor.

lipid composition comes very close to that found in our plasmalemmal fraction. Nevertheless, all these fractions retain distinctive enzymatic activities (45). Substantial amounts of sphingomyelin and cholesterol have been recorded in other secretory granules, such as the catecholamine-containing granules of the adrenal medulla obtained from a variety of species (26, 27), as well as the small and large secretory granules isolated from the bovine anterior pituitary (50). In all these cases it is presumed that these lipids are components of the limiting membrane of the corresponding granules. The catecholamine granules of a number of species also contain lysolecithin in relatively high concentration as shown by Blashko et al. (26, 27), who assume that the lysophosphatide plays a role in the fusion of the secretory granule membrane with the cell membrane prior

to discharge. Our data put in doubt the presence of large amounts of lysolecithin in zymogen granule membranes *in vivo*, and hence cannot provide unquestionable support for the hypothesis mentioned.

A high sphingomyelin and cholesterol content has been reported also for the limiting membranes of hepatic lysosomes (51), i.e., cell components which, to a certain extent, behave like zymogen granules in their relationship to the plasmalemma and the Golgi complex.

Finally, the lipid composition of our pancreatic plasmalemmal fraction is quite similar to that reported in the literature for cell membrane fractions prepared from other sources. With few exceptions which could be ascribed to heavy microsomal contamination (52), sphingomyelin has been found in high concentration in plasmalemmal fractions isolated from liver homogenates (51, 53-58) (range from 14% [55] to 33% [56] of total phospholipids) and erythrocytes (59, 60). The same applies for cholesterol which is found in high concentrations in cell membrane fractions prepared from liver (51-58, 61, 62), intestinal brush borders (61, 63, 64), erythrocytes,⁹ (59, 61), kidney (62), urinary bladder (65), and HeLa cells (66).

The pancreatic plasmalemmal fractions contain glycolipids among which ceramide dihexoside is the predominant species. At lower concentrations, this compound was encountered in all the other membrane fractions we studied. Gangliosides were not detected in pancreatic membrane fractions. Glycolipids have been identified but not fully characterized in plasmalemmal fractions from a variety of sources (53, 54, 56, 58, 60, 64). In liver, they are 10 times more concentrated in this fraction than in microsomes and the predominant species is ceramide monohexoside (56, 67). The hepatic plasmalemmal fractions also contain traces of gangliosides (56, 67). Large amounts of gangliosides and cerebroside have been found in erythrocyte membranes (60) and some cerebroside have been detected in the secretory granules of the anterior pituitary (50).

Considered in their broadest aspect, our results indicate that a clear distinction can be made on

⁹ The molar ratios cholesterol/phospholipid reach from 0.4 (58) to 0.89 (58), from 0.46 (61) to 1.26 (64), and from 0.72 to 1.1 (59) for cell membrane fractions isolated from liver, intestinal brush borders, and erythrocytes, respectively.

TABLE X
Lipid Composition of Pancreatic Membrane Fractions

	Rough microsomal membranes	Smooth microsomal membranes	Zymogen granule membranes	Plasma membranes
PLP/total lipids-FFA	0.84	0.75	0.71	0.71
Sphingomyelin (% total PLP)	3.4	14.2	23.5	19.2
Lecithin + lysolecithin (% total PLP)	47.8	39.4	30.5	32.0
Ph-ethanolamine (% total PLP)	35.8	36.5	31.5	34.4
Ph-serine + Ph-inositol (% total PLP)	5.6	4.7	5.0	4.6
Front (% total PLP)	4.2	3.5	5.9	4.6
Total cholesterol (μ mole/ μ mole PLP)	0.12	0.47	0.55	0.51

The data given in Tables I and II have been corrected by: (a) deducting FFA amounts from total lipids, and (b) adding lysolecithin to the lecithin column.

the basis of lipid composition between the membrane of the rough-surfaced endoplasmic reticulum (rough microsomes), which is rich in lecithin but poor in sphingomyelin and cholesterol, and the membranes of the Golgi complex (smooth microsomes), zymogen granules, and cell surface which are relatively rich in these lipids. Further differences, especially among membranes of the smooth type, are indicated by a survey of their enzymatic activities (45). For the moment, it is clear that transport of secretory products from the rough ER to the Golgi complex must be carried out in vivo by a mechanism which does not lead to membrane mixing, but allows each of the membranes involved to retain its characteristic lipid composition.

This investigation was supported by Public Health Service Research Grants AM-10928 and HE-05648 from the National Institutes of Health.

Received for publication 20 July 1970, and in revised form 14 October 1970.

REFERENCES

- MELDOLESI, J., J. D. JAMIESON, and G. E. PALADE. 1971. *J. Cell Biol.* **49**:109.
- BLOOR, W. R. 1943. In *Biochemistry of Fatty Acids*. Reinhold Publishing Corporation, New York. 70.
- SIEKEVITZ, P., and G. E. PALADE. 1958. *J. Biophys. Biochem. Cytol.* **4**:203.
- DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. *Biochem. J.* **60**:604.
- FOLCH, J., M. LEES, and G. H. SLOANE-STANLEY. 1957. *J. Biol. Chem.* **266**:497.
- SKIPSKI, V. P., R. F. PATTERSON, and M. BARCLAY. 1964. *Biochem. J.* **90**:374.
- SKIPSKI, V. P., J. J. GOOD, M. BARCLAY, and R. B. REGGIO. 1968. *Biochim. Biophys. Acta.* **152**:10.
- SKIPSKI, V. P., A. F. SMOLOWE, and M. BARCLAY. 1967. *J. Lipid Res.* **8**:295.
- LEDEEN, R., K. SALSMAN, and M. CABRERA. 1968. *Biochemistry.* **17**:2287.
- SIMS, R. P. A., and J. A. G. LAROSE. 1962. *J. Amer. Oil Chem. Soc.* **39**:232.
- MANGOLD, H. K. 1961. *J. Amer. Oil Chem. Soc.* **38**:708.
- SVENNERHOLM, L. 1956. *J. Neurochem.* **1**:42.
- SVENNERHOLM, L. 1957. *Biochim. Biophys. Acta.* **24**:604.
- GOLDRICK, B., and J. HIRSCH. 1963. *J. Lipid Res.* **4**:482.
- AMES, B. 1966. In *Methods in Enzymology*. E. F. Neufeld and V. Ginsburg, editors. Academic Press Inc., New York. **8**:115.
- AMENTA, J. S. 1964. *J. Lipid Res.* **5**:270.
- BENZONANA, G. 1968. *Biochim. Biophys. Acta.* **151**:137.
- BRAY, G. A. 1960. *Anal. Biochem.* **1**:279.
- DE HAAS, G. H., N. M. POSTEMA, W. NIEUWENHUIZEN, and L. L. M. VAN DEENEN. 1968. *Biochim. Biophys. Acta.* **159**:103.
- FLEISCHER, S., and G. ROUSER. 1965. *J. Amer. Oil Chem. Soc.* **42**:588.
- ROUSER, G., G. J. NELSON, S. FLEISCHER, and G. SIMON. 1968. In *Biological Membranes, Physical Fact and Function*. D. Chapman, editor. Academic Press Inc., New York. 5.
- LEIKOLA, E., E. NIEMINEN, and E. SALOMAA. 1965. *J. Lipid Res.* **6**:490.
- SALDIN, T. A., and E. A. NAPIER, JR. 1967. *J. Lipid Res.* **8**:342.

24. LOUGH, A. K., and G. A. GARTON. 1968. *Lipids*. 3:321.
25. LOUGH, A. K., L. FELINSKI, and G. A. GARTON. 1962. *J. Lipid Res.* 3:478.
26. BLASHKO, H., H. FIREMARK, A. D. SMITH, and H. WINKLER. 1967. *Biochem. J.* 104:545.
27. WINKLER, H., N. STRIEDER, and E. ZIEGLER. 1967. *Arch. Pharmakol. Exp. Pathol.* 256:407.
28. PROTTEY, C., and J. N. HAWTHORNE. 1966. *Biochem. J.* 101:191.
29. GLAUMANN, H., and G. DALLNER. 1968. *J. Lipid Res.* 9:720.
30. TRUMP, B. F., A. U. ARSTILA, S. M. DUTTERA, and W. L. BYRNE. 1969. *Fed. Proc.* 28:403.
31. FINEAN, J. B., and A. MARTONOSI. 1965. *Biochim. Biophys. Acta.* 98:547.
32. JAMIESON, J. D. 1966. Ph.D. Thesis. The Rockefeller University, New York.
33. DE HAAS, G. H., N. M. POSTEMA, W. NIEUWENHUIZEN, and L. L. M. VAN DEENEN. 1968. *Biochim. Biophys. Acta.* 159:118.
34. VAN DEN BOSCH, H., N. M. POSTEMA, G. H. DE HAAS, and L. L. M. VAN DEENEN. 1965. *Biochim. Biophys. Acta.* 98:657.
35. MAGEE, W. L., J. GALLAY-HATCHARD, H. SANDERS, and R. H. S. THOMPSON. 1962. *Biochem. J.* 83:17.
36. DESNUELLE, P., L. SARDA, and G. AILHAUD. 1960. *Biochim. Biophys. Acta.* 37:571.
37. WILLS, E. D. 1960. *Biochim. Biophys. Acta.* 40:481.
38. DE HAAS, G. H., L. SARDA, and J. ROGER. 1965. *Biochim. Biophys. Acta.* 106:638.
39. CHEN, R. F. 1967. *J. Biol. Chem.* 242:173.
40. ROUSER, G., G. FELDMAN, and C. GALLI. 1965. *J. Amer. Oil Chem. Soc.* 42:411.
41. GETZ, G. S., W. BARTLEY, D. LURIE, and B. M. NOTTON. 1968. *Biochim. Biophys. Acta.* 152:325.
42. MARINETTI, G. V., J. ERBLAND, and E. STOLTZ. 1958. *J. Biol. Chem.* 233:562.
43. MARTONOSI, A. 1964. *Fed. Proc.* 23:913.
44. TSAO, S. S., and W. E. CORNATZER. 1967. *Lipids.* 2:41.
45. MELDOLESI, J., J. D. JAMIESON, and G. E. PALADE. 1971. *J. Cell Biol.* 49:150.
46. JAMIESON, J. D., and G. E. PALADE. 1967. *J. Cell Biol.* 34:577.
47. KEENAN, T. W., and J. D. MORRÉ. 1970. *Biochemistry.* 9:19.
48. EHRENREICH, J. H. 1969. Ph.D. Thesis. The Rockefeller University, New York.
49. FLEISCHER, B., S. FLEISCHER, and H. OZAWA. 1969. *J. Cell Biol.* 43:59.
50. TESAR, J. T. 1967. *Fed. Proc.* 26:534.
51. THINÈS-SEMPOUX, D. 1967. *Biochem. J.* 105:20 P.
52. TAKEUCHI, M., and H. TERAYAMA. 1965. *Exp. Cell Res.* 40:32.
53. RAY, T. K., V. P. SKIPSKI, M. BARCLAY, E. ESSNER, and F. M. ARCHIBALD. 1969. *J. Biol. Chem.* 244:5528.
54. COLEMAN, R., R. H. MICHELL, J. B. FINEAN, and J. N. HAWTHORNE. 1968. *Biochim. Biophys. Acta.* 135:573.
55. STAHL, W. L., and E. G. TRAMS. 1968. *Biochim. Biophys. Acta.* 163:459.
56. DOD, B. J., and G. M. GRAY. 1968. *Biochim. Biophys. Acta.* 150:397.
57. PFLEGER, R. C., N. G. ANDERSON, and F. SNYDER. 1968. *Biochemistry.* 7:2826.
58. BENEDETTI, E. L., and P. EMMELOT. 1968. In *The Membranes*. A. J. Dalton and F. Hagenau, editors. Academic Press Inc., New York. 33.
59. DE GIER, J., and L. L. M. VAN DEENEN. 1961. *Biochim. Biophys. Acta.* 49:268.
60. NELSON, G. J. 1967. *Biochim. Biophys. Acta.* 144:221.
61. ASHWORTH, L. A. E., and C. GREEN. 1966. *Science (Washington).* 151:210.
62. COLEMAN, R., and J. B. FINEAN. 1966. *Biochim. Biophys. Acta.* 125:197.
63. EICHHOLZ, A. 1967. *Biochim. Biophys. Acta.* 135:475.
64. FORSTNER, G. G., K. TANAKA, and K. J. ISSELBACHER. 1968. *Biochem. J.* 109:51.
65. HICKS, R. M., B. KETTERER, and D. BEALE. 1968. *Biochem. J.* 109:41 P.
66. BORMAN, H. B., A. HAGOPIAN, and E. H. EYLAR. 1968. *Arch. Biochem. Biophys.* 128:51.
67. DOD, B. J., and G. M. GRAY. 1968. *Biochem. J.* 110:50 P.