Lipid Composition of the Isolated Rat Intestinal Microvillus Membrane

By G. G. FORSTNER,* K. TANAKA AND K. J. ISSELBACHER

Department of Medicine, Harvard Medical School and the Medical Services (Gastrointestinal Unit),
Massachusetts General Hospital, Boston, Mass. 02114, U.S.A.

(Received 10 January 1968)

1. Rat intestinal microvillus plasma membranes were prepared from previously isolated brush borders and the lipid composition was analysed. 2. The molar ratio of cholesterol to phospholipid was greatest in the membranes and closely resembled that reported for myelin. 3. Unesterified cholesterol was the major neutral lipid. However, 30% of the neutral lipid fraction was accounted for by glycerides and fatty acid. 4. Five phospholipid components were identified and measured, including phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, sphingomyelin and lysophosphatidylcholine. Though phosphatidylethanolamine was the chief phospholipid, no plasmalogen was detected. 5. In contrast with other plasma membranes in the rat, the polar lipids of the microvillus membrane were rich in glycolipid. The cholesterol:polar lipid (phospholipid + glycolipid) ratio was about 1:3 for the microvillus membrane. Published data suggest that this ratio resembles that of the liver plasma membrane more closely than myelin or the erythrocyte membrane. 6. The fatty acid composition of membrane lipids was altered markedly by a single feeding of safflower oil. Membrane polar lipids did not contain significantly more saturated fatty acids than cellular polar lipids. Differences in the proportion of some fatty acids in membrane and cellular glycerides were noted. These differences may reflect the presence of specific membrane glycerides.

The intestinal microvillus plasma membrane lines the lumen of the upper gut and is the first absorptive surface to be permeated by most nutrient foodstuffs. Previous work has established this membrane as the site of many hydrolytic enzymes (Eichholz & Crane, 1965; Forstner, Sabesin & Isselbacher, 1968; Johnson, 1967) and several transport carrier systems (Kinter & Wilson, 1965). Histochemically it has been shown to be rich in carbohydrate (Rambourg, Neutra & Leblond, 1966) and chemical analysis has indicated that it possesses, in common with several other plasma membranes, a high proportion of cholesterol in relation to phospholipid (Finean, Coleman & Green, 1966; Coleman & Finean, 1966; Ashworth & Green, 1966; Eichholz, 1967).

In a previous paper a highly reproducible method for the preparation of rat intestinal microvillus membranes from isolated intestinal brush borders was described (Forstner *et al.* 1968). The preparation is free of significant contamination by either fibrillar material of the brush border or nuclear debris, and is therefore suitable for a detailed examination of the plasma membrane. In the present paper details of the lipid composition

of isolated microvillus plasma membranes are reported, and the results are examined for their relevance to intestinal function.

MATERIALS AND METHODS

Membrane preparation. Microvillus membranes were prepared from rat intestinal brush borders as described by Forstner *et al.* (1968).

Extraction of lipid. Lipids were extracted as described by Folch, Lees & Sloane-Stanley (1956). Lower and upper phases were allowed to separate by standing overnight at 4°. The lower phase was washed three times with upper-phase solvents containing KCl (0.37%) until all interphase material was removed. The lower phase was then evaporated to dryness under N₂ at 23° and stored in chloroform under N₂. When weight determinations were to be made, the total extraction procedure was performed twice.

Solvents. All solvents were 'Baker Analyzed' and obtained from Howe and French, Boston, Mass., U.S.A. Chloroform was washed with H₂SO₄ and then distilled water, and dried over anhydrous Na₂SO₄ and redistilled.

Separation of neutral and phospholipids. Lipids in 1–2 ml. of chloroform were applied to columns containing 2 or 4 g. of silicic acid (100–200 mesh; Calbiochem, Los Angeles, Calif., U.S.A.), activated at 120° for 24 hr. (Börgstrom, 1952). Approx. 1 g. of silicic acid was used/mg. of lipid. Neutral lipids were eluted with 50 ml. of chloroform, and phospholipids with 50 ml. of methanol. A flow rate of 2–2½ ml./min. was maintained.

* Present address: Department of Medicine, University of Toronto, Toronto, Ont., Canada.

Separation and estimation of neutral lipids. Neutral lipids were applied in a horizontal series of spots to glass plates coated with a 0.25 mm. layer of silica gel G (E. Merck A.-G., Darmstadt, Germany). The coated plates were activated for 1 hr. at 105°. Lipid standards were spotted adjacent to the unknown lipid bands. Esterified cholesterol, triglyceride, fatty acid and a band containing monoglyceride, diglyceride and cholesterol were separated in light petroleum (b.p. 60–80°)-diethyl ether-acetic acid (6:1:1, by vol.). The spots to be analysed were protected with aluminium foil, and parallel reference standards developed with iodine vapour. The aluminium foil was then removed and the lipid areas were carefully scraped into 50 ml. centrifuge tubes. After elution with three 15 ml. volumes of chloroform, the solvent was evaporated under N₂. Cholesterol, diglycerides and monoglycerides were separated further on silica gel G in benzene-diethyl ether-acetic acid (50:50:1, by vol.) (Heimberg, Weinstein, Dishmon & Fried, 1965) and treated similarly.

Cholesterol was determined by the method of Zlatkis, Zak & Boyle (1953). Glycerides were determined by the method of Van Handel & Zilversmit (1957). Fatty acids were determined by the method of Dole (1956).

Separation and estimation of phospholipids. Mild alkaline and acid hydrolysis of phospholipids, and subsequent separation and detection of water-soluble phosphates, was carried out as described by Dawson (1960). Hydrolysates were separated by descending chromatography on Whatman no. 1 paper previously washed with 2*N*-acetic acid and water. The chromatograms were developed for 14–18 hr. with freshly prepared redistilled phenol saturated with water-acetic acid-ethanol (50:5:6, by vol.). Hydrolysed reference standards were prepared from phospholipids previously purified by thin-layer chromatography. Standards of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were shown to have *R_F* values 0.85, 0.65, 0.34 and 0.23 respectively. Phosphorus-containing spots and intervening areas were cut out and analysed for P by the method of Bartlett (1959) as modified by Marinetti (1962), after hydrolysis in 2.0 ml. of 70% (w/v) HClO₄.

Unhydrolysed phospholipids were separated and quantitatively analysed by two-dimensional thin-layer chromatography as described by Abramson & Blecher (1964). Phosphorus-containing areas were identified with the spray reagent described by Dittmer & Lester (1964). Phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine were detected by means of the reagents described by Skidmore & Entenman (1962).

Separation and estimation of glycolipids. Glycolipids in methanol eluates of silicic acid columns were separated by one-dimensional thin-layer chromatography on silica gel G (E. Merck A.-G. in chloroform-methanol-water (65:25:4, by vol.) (Wherrett, 1967). Glycolipids were detected with aniline-diphenylamine (Harris & MacWilliams, 1954) and orcinol-HCl sprays. Total glycolipid was estimated as the difference in weight between total lipid and the sum of neutral lipid and phospholipid. Lipid hexose was determined with anthrone (Wells & Dittmer, 1963).

Estimation of fatty acids. Fatty acids were estimated by gas-liquid chromatography after hydrolysis of esterified lipids and methylation with methanolic H₂SO₄ (Lipsky & Landowne, 1963). A Barber-Colman model 5000 gas-liquid chromatograph equipped with a hydrogen flame detector was used. The glass U-tube (4 mm. diam. × 6 ft. long) was packed with a mixture of 5% (w/w) ethylene glycol succinate (Applied Science Laboratories, State College, Pa., U.S.A.) and Anakrom A (80–90 mesh; Analytical Engineering Laboratories, Hamden, Conn., U.S.A.), and N₂ was used as carrier with an inlet pressure of 20 lb./in.². The temperatures of the column, flash evaporator and detector were 175°, 240° and 220° respectively.

RESULTS

Microvillus membrane cholesterol and phospholipid.

In the initial evaluation of the method for isolating microvillus membranes, phospholipid and cholesterol determinations were employed as markers of the membrane content of the various fractions obtained by disrupting intestinal brush borders (Forstner *et al.* 1968). In Table 1 the cholesterol and phospholipid content of our brush border and microvillus membrane preparations is shown. The isolated membrane contained more cholesterol and phospholipid/mg. of protein than the brush border. The major increase was in the cholesterol content, however, as the cholesterol:phospholipid molar ratio of the membrane preparation was greater than that of the brush border.

Phospholipid composition. The phospholipid fraction obtained from three separate membrane preparations was examined by two-dimensional chromatography and the individual phospholipid spots were analysed quantitatively. Only five

Table 1. *Cholesterol and phospholipid content of the brush border and microvillus membrane of rat intestine*

Cholesterol and phospholipid were determined as described in the Materials and Methods Section. The results are expressed as means ± S.D.; the numbers of preparations analysed are given in parentheses.

Preparation	Cholesterol (mg./100 mg. of protein)	Phospholipid (mg./100 mg. of protein)	Cholesterol	
			(mg./100 mg. of phospholipid)	(moles/mole of phospholipid)
Brush border	5.3 ± 0.6 (5)	10.2 ± 0.7 (5)	52.0 ± 7.0 (5)	1.0
Microvillus membrane	8.5 ± 0.5 (7)	13.3 ± 0.8 (7)	64.3 ± 4.3 (7)	1.26

Table 2. *Phospholipid content of microvillus membrane*

Phospholipids were analysed by two-dimensional thin-layer chromatography as described in the Materials and Methods section. Each determination refers to the results obtained with a separate membrane preparation.

Phospholipid	No. of determinations	Phospholipid content (%)	
		Mean	Range
Phosphatidylethanolamine	3	39.5	35.0-43.0
Phosphatidylcholine	3	20.7	13.5-28.0
Phosphatidylserine	3	14.0	13.5-15.0
Sphingomyelin	3	6.7	2.5-15.0
Lysophosphatidylcholine	3	1.7	0.5-4.2

phosphorus-containing spots were found, corresponding to phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, sphingomyelin and lysophosphatidylcholine. As shown in Table 2, phosphatidylethanolamine and phosphatidylcholine were the major phospholipids. Phosphatidylserine and phosphatidylethanolamine accounted for more than 50% of the total phospholipid. Phosphatidylinositol, which migrates more rapidly than either of these phospholipids or phosphatidylcholine in the second solvent system employed, was not identified. Considerable difficulty was encountered in obtaining satisfactory elution of the choline-containing spots from silicic acid, with the result that values obtained for these phospholipids varied widely. However, even when the maximal values were taken into account it was evident that the microvillus membrane contained more acidic phospholipids.

The individual membrane preparations examined in these experiments contained only 0.6-0.8 mg. of phospholipid, and were therefore insufficient for duplicate analyses, or satisfactory weighing. To avoid the difficulties encountered in dealing with small quantities of lipids and to permit the examination of membrane phospholipids by an alternative method, five separate preparations, each representing the microvillus membrane obtained from 12 rats, were accumulated and combined. The pooled membranes possessed a mean invertase specific activity of 1.67 ± 0.22 ($\pm$ s.d.), and contained 8.4 mg. of cholesterol and 14.3 mg. of phospholipids/100 mg. of protein. These values correspond very closely to those previously established as characteristic of highly pure microvillus membranes (Table 1 in Forstner *et al.* 1968).

The accumulated membrane lipid contained 3.78 mg. of phospholipid, which was analysed by mild alkaline and acid hydrolysis (as described in the Materials and Methods section) after separation from neutral lipid. The results of this analysis are shown in Tables 3 and 4. With alkali, 78.8% of the phospholipid was hydrolysed, whereas negligible hydrolysis occurred in dilute acid. Acid-labile phospholipid was not detected by the analytical

Table 3. *Fractionation of microvillus membrane phospholipid by mild alkaline and acid hydrolysis*

Acid and alkaline hydrolysis were carried out by the method of Dawson (1960). —, None detected.

Fraction	Phospholipid		
	(mg.)	(%)	(mg./mg. of protein)
Total phospholipid	3.78	100	0.143
Alkali-labile	2.98	78.8	0.113
Acid-labile	—	—	—
Alkali- and acid-stable	0.63	16.6	0.024
Recovery	3.62	95.5	

Table 4. *Quantitative paper chromatography of alkali-labile hydrolysis products*

Hydrolysis products	% of	
	alkali-labile phospholipid	% of phospholipid
Phosphatidylethanolamine	41	32.2
Phosphatidylcholine	35	27.7
Phosphatidylserine	13.5	10.7
Other (R_F 0.24)	10.4	8.2

methods used, but was less than 0.2% of the total since the remainder of the alkali-stable phospholipids were recovered in the alkali- and acid-resistant residue. In concurrent experiments, with a lipid fraction from whole brush borders, 85% of the phospholipid was found to be alkali-labile and none was acid-labile. Alkali- and acid-stable phospholipid was not characterized further. The major component of this fraction is sphingomyelin, though other phospholipids including ethanolamine-containing phosphatides may be present (Dawson, 1960).

Three spots corresponding to the hydrolysis products of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine were found in paper chromatograms of the alkaline hydrolysate. A fourth spot, with R_F 0.24 in the solvent system

used, was assumed to be inorganic phosphate. Phosphatidylinositol has a similar R_F , but was not identified by staining with ammoniacal silver nitrate (Skidmore & Entenman, 1962). As indicated in Table 4, serine- and ethanolamine-containing phospholipids accounted for 43% of the total lipid phosphorus, and choline-containing phospholipids other than sphingomyelin accounted for 27%. The results obtained by the hydrolytic technique agree with those obtained by thin-layer chromatography in demonstrating a simple phospholipid composition including only ethanolamine-, choline- and serine-containing phosphatides.

Neutral-lipid composition. The neutral lipid fraction obtained from the pooled membrane was sufficiently large to permit quantitative determination of fatty acid and glyceride components.

As shown in Table 5, the neutral lipid fraction determined by chemical analysis of individual components after their separation by thin-layer chromatography amounted to 3.15mg. Of the neutral lipid 70.5% was cholesterol, and none of this was esterified. Of the remaining lipids in this fraction, fatty acids and diglycerides accounted respectively for 13.0 and 11.0% of the total; only small amounts of mono- and tri-glycerides were found.

Table 5. *Neutral-lipid composition of microvillus membrane*

Neutral lipids were determined as described in the Materials and Methods section. Total neutral lipid refers to the sum of the separately determined fractions.

Fraction	Amount of neutral lipid		
	(mg.)	(%)	(mg./mg. of protein)
Total neutral lipid	3.15	100	0.119
Cholesterol	2.21	70.5	0.084
Fatty acid	0.42	13.0	0.016
Monoglyceride	0.08	2.5	0.003
Diglyceride	0.34	11.0	0.013
Triglyceride	0.10	3.0	0.004

Other lipids. The availability of relatively large amounts of microvillus membrane made it possible to determine the dry weight of the major lipid fractions for the first time. From a membrane fraction containing a total of 26.4mg. of protein 16.2mg. of lipid was extracted. As indicated in Table 6, only 43% of the total lipid could be accounted for by chemical determination of neutral lipids and phospholipids. The discrepancy was not due to loss of lipid during silicic acid fractionation since the combined recovery of lipid in the eluates from the column was approx. 100%.

In view of the fact that most of the unaccountable lipid was present in the methanol eluate it seemed likely that the microvillus membrane might contain large quantities of glycolipid. Two-dimensional chromatograms that were sprayed with orcinol-hydrochloric acid revealed at least five reactive spots. Since all other lipids identified by iodine vapour contained phosphorus, most of the remaining lipid in the microvillus membrane appeared to be glycolipid. By one-dimensional chromatography the membrane glycolipids were tentatively identified as cerebroside, ceramide dihexoside, ceramide trihexoside or sulphatide (or both), and several gangliosides.

In three membrane preparations (Table 7) the sum of the total neutral lipid and phospholipid fractions estimated chemically accounted for less than 50% of the total lipid weight. In two preparations evidence that most of the remaining lipid was glycolipid was derived from estimation of hexose. On the assumption that the molecular weight of membrane glycolipids is 846 or 4.7 times the molecular weight of galactose (Autilio, Norton & Terry, 1964), total glycolipid was estimated and found to agree with estimates arrived at by weight difference. Since these membranes were derived in part from the aqueous phase above sucrose gradients (membrane II; Forstner *et al.* 1968) the possibility was considered that some contamination of the lipid with sucrose may have occurred even after the membranes were washed in 10 vol. of 2.5mM-EDTA and twice extracted. In control experiments, however, in which the aqueous phase from gradients

Table 6. *Identification and recovery of membrane lipid in fractions separated by silicic acid chromatography*

Microvillus membrane lipid (16.2 mg.) was applied to an activated silicic acid column, and eluted with chloroform and then methanol. Total neutral lipids in the chloroform eluate were determined as in Table 5 and total phospholipids in the methanol eluate as described in the Materials and Methods section.

Membrane lipids	Chloroform eluate (mg.)	Methanol eluate (mg.)	Recovery	
			(mg.)	(% of total)
Total lipid (dry wt.)	4.7	12.6	17.3	105
Neutral lipid	3.2	3.8	7.0	43
Phospholipid	—			

Table 7. *Neutral lipid, phospholipid and glycolipid as percentages of total lipid (dry weight) in three microvillus membrane preparations*

Data on membrane fraction 1 are taken from Table 6. Neutral lipid in membrane preparations 2 and 3 (calculated from the cholesterol content) was 13.7% of the total lipid dry weight in each case. It was assumed that microvillus membrane neutral lipid is 70% cholesterol. Estimates of glycolipid content derived by weight difference and by hexose assay are compared. Total glycolipid was calculated from the hexose content on the assumption that mixed glycolipids have a molecular weight of 846 (Autilio *et al.* 1964).

Membrane preparation	Lipids								
	Total (mg.)	Neutral lipid		Phospholipid		Glycolipid (weight)		Glycolipid (hexose)	
		(mg.)	(% of total)	(mg.)	(% of total)	(mg.)	(% of total)	(mg.)	(% of total)
1	16.2	3.15	19.5	3.78	23.3	9.27	57.0	—	—
2	11.7	2.3	19.5	2.92	25.0	6.48	55.0	6.78	58.0
3	6.0	1.16	19.5	1.79	29.8	3.05	51.0	2.9	48.5

Table 8. *Fatty acid composition of microvillus membrane and 'cellular' lipids*

The 'cellular' fraction refers to the supernatant obtained after centrifuging the initial mucosal homogenate at 400 *g* for 10 min. to remove brush borders. Lipids were extracted, separated and analysed as described in the Materials and Methods section. Rats were starved for 16 hr. before being killed. —, None detected.

		Fatty acid composition (%)								
Lipids	Cell fraction	C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:3}	C _{20:4}
'Polar'	Microvillus membrane	0.5	15.6	0.8	29.5	5.8	14.4	1.3	2.3	22.0
	'Cellular'	0.4	18.1	0.7	22.9	9.8	19.8	—	—	28.4
Triglyceride	Microvillus membrane	0.6	15.2	1.7	12.3	15.4	30.5	1.6	—	22.6
	'Cellular'	0.4	20.2	1.3	13.9	19.2	24.9	0.8	—	18.0
Diglyceride	Microvillus membrane	0.6	28.1	0.8	34.8	21.6	8.7	—	—	5.5
	'Cellular'	0.4	23.2	1.6	23.0	13.1	22.9	0.8	—	14.8

was added to samples of homogenate and carried through the preparative washings and extractions, no contamination with sucrose was found.

Fatty acid composition. The fatty acids present in three lipid fractions isolated from microvillus membrane are shown in Tables 8 and 9. In these experiments membrane lipids were compared with those of the 'cellular' supernatant (essentially free of microvillus membrane) obtained from the initial centrifugation of the intestinal mucosal homogenate. Since microvillus membrane and cellular lipids might differ in their ability to incorporate dietary fatty acids, lipids were examined from rats that had been starved for 16 hr. (Table 8) and from rats that had received 3 ml. of safflower oil by stomach tube 3 hr. before being killed (Table 9). Lipids were separated by silicic acid chromatography into a 'polar' fraction, containing phospholipids and glycolipids, and a neutral lipid fraction. Diglycerides and triglycerides were obtained by thin-layer chromatography of the neutral lipid fractions and elution of the glyceride spots with chloroform.

As shown in Table 8, under conditions of starvation both membrane and cellular polar lipid fractions contained slightly more polyunsaturated than saturated fatty acid and little mono-unsaturated fatty acid. The fatty acid composition of each glyceride fraction possessed distinctive features. Triglycerides contained a large amount of linoleic acid and a preponderance of unsaturated acids; diglycerides were rich in palmitic acid and stearic acid. Though no major differences were apparent between membrane and cellular triglyceride fatty acids, membrane diglycerides contained less linoleic acid and arachidonic acid and more oleic acid, stearic acid and palmitic acid. All three lipid fractions appeared to incorporate fatty acids supplied by safflower oil, in that each became enriched with linoleic acid after this oil was given (Table 9). The incorporation of safflower oil fatty acids was most marked in the cellular glycerides; these had a fatty acid composition almost identical with that of safflower oil. Less marked enrichment with linoleic acid was noted in both membrane glyceride fractions. The diglyceride fraction of the

Table 9. *Effect of giving safflower oil on the fatty acid composition of microvillus membrane and 'cellular' lipids*

Rats were given 3.0 ml. of safflower oil by stomach tube 3 hr. before being killed. Tissue fractions and analytical methods are as described in Table 8. —, None detected.

Lipid	Cell fraction	Fatty acid composition (%)							
		C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:3}
Phospholipid	Microvillus membrane	1.4	13.3	2.5	19.2	26.3	29.4	0.3	—
	'Cellular'	0.1	17.3	0.3	19.2	11.1	38.6	0.4	5.1
Triglyceride	Microvillus membrane	2.3	16.0	2.7	4.6	18.9	55.5	—	—
	'Cellular'	—	7.6	—	1.4	11.8	78.2	0.9	—
Diglyceride	Microvillus membrane	0.1	8.9	0.7	25.3	19.0	40.6	0.9	—
	'Cellular'	—	8.4	—	4.1	15.0	72.5	—	4.6
Safflower oil		—	7.3	—	2.3	14.4	76.0	—	—

Table 10. *Major lipid components of plasma membranes of the rat*

Membrane	Reference	Neutral lipid (%)	Phospholipid (%)	Glycolipid (%)
Intestinal microvillus	Present authors	20.0	26	54
Liver cell	Skipski <i>et al.</i> (1965)	28.3	39	26.4
Myelin	Autilio <i>et al.</i> (1964)	26.4	42.8	29.7*
Erythrocyte	de Gier & van Deenen (1961)	28.0	61.0	11.0

* Determined as galactose, assuming an average molecular weight of 846 for rat brain glycolipid. In the remaining instances glycolipid was estimated as the difference in weight between total lipid and the sum of neutral lipid and phospholipid.

membrane lipid also contained a large stearic acid component that was lacking in the cellular diglyceride. Membrane polar lipids contain less arachidonic acid and linoleic acid but more oleic acid than cellular polar lipids. In all three lipid fractions linoleic acid incorporation was associated with a decrease in saturated fatty acid and arachidonic acid, whereas oleic acid usually increased.

DISCUSSION

The present results indicate that the microvillus membrane of the rat intestine contains approx. 600 µg. of lipid/mg. of protein. Of the total lipid approx. 20% is neutral lipid, approx. 30% is phospholipid and as much as 50% may be glycolipid. The high cholesterol:phospholipid molar ratio found in our microvillus preparation (Table 1) confirms previous observations made by Finean *et al.* (1966) and Coleman & Finean (1966) in the guinea pig, and by Eichholz (1967) in the hamster. The ratio 1.26 is comparable with that reported for myelin by Autilio *et al.* (1964) and is slightly greater than values reported for erythrocyte membrane (Coleman & Finean, 1966; Ashworth & Green, 1966). Other plasma membranes appear to possess much smaller ratios (Millington & Finean, 1963; Coleman & Finean, 1966; Ashworth & Green,

1966), but Coleman & Finean (1966) have shown that the cholesterol:phospholipid ratio rose in plasma membranes from different tissues in relation to the degree of purification. A high ratio may therefore become apparent as methods for isolating a particular plasma membrane are improved. In Table 1 it is clear that the purification of the microvillus plasma membrane associated with its isolation from whole brush borders has resulted in a considerable increment in the cholesterol:phospholipid ratio.

Finean (1953) and van Deenen, Houtsmuller, de Haas & Mulder (1962) have suggested that a high cholesterol:phospholipid ratio might promote order and rigidity in cell membranes and could account for the stability of myelin and the erythrocyte membrane. The stability of the microvillus membrane has not been adequately studied, but a high resistance to hypo-osmotic shock has been reported (Millington & Finean, 1962).

The significance of the cholesterol:phospholipid ratio in plasma membranes may require re-evaluation, however, in view of the present findings that most of the polar lipids in the microvillus plasma membrane may be glycolipids. The interaction of cholesterol and glycolipid appears not to have been investigated to date, but it is likely that glycolipids may closely resemble

phospholipids in their behaviour. Assuming that weight difference determinations adequately estimate total glycolipid, and that the mean molecular weight of glycolipid is 846, it can be calculated from the data in Table 7 that cholesterol, phospholipid and glycolipid are present in the membrane in the approximate molar proportions 1:1:2. The cholesterol:polar lipid ratio is thus 1:3 in the microvillus membrane. Calculations from the data (Table 10) of Skipski *et al.* (1965), de Gier & van Deenen (1961) and Autilio *et al.* (1964) indicate that the liver plasma membrane has a similar cholesterol:polar lipid ratio (approx. 3:8), but in both the erythrocyte and myelin membranes the ratios are close to unity. Thus when cholesterol is considered in relation to total lipid the four plasma membranes for which data are available appear to fall in two distinct groups.

In the present study the only phospholipids identified by the techniques employed were phosphatidylethanolamine, lecithin, phosphatidylserine, sphingomyelin and lysolecithin (Tables 2 and 3). Kephals were found to represent 43 and 53.5% of the total phospholipids by paper chromatography of alkaline hydrolysates and thin-layer chromatography of intact phospholipids (Tables 2 and 4). In its high content of kephalin the microvillus membrane most resembles rat myelin (Nussbaum, Bieth & Mandel, 1963), whereas a preponderance of lecithin has been found in both the rat erythrocyte membrane (de Gier & van Deenen, 1961) and the liver plasma membrane (Skipski *et al.* 1965). Since phosphatidylethanolamine and phosphatidylserine have been shown to be capable of directing cation movement in model systems (Hoffman, Schulman & Eden, 1959) and the addition of phosphatidylethanolamine increases the permeability of myelinoid lecithin dispersions (Fast, 1967), these differences in kephalin:lecithin ratios in the various plasma membranes of the rat may be of functional significance.

The absence of acid-hydrolysable phospholipids suggests that the microvillus membrane contains very little plasmalogen phosphatide. The inability to demonstrate plasmalogen is noteworthy, since kephals, particularly phosphatidylethanolamine, are often rich in plasmalogens (Webster, 1960; Marcus, Ullman & Wolfman, 1960). Plasmalogens have not apparently been determined in rat liver plasma membrane, but account for 30% of the phospholipid in rat myelin (Autilio *et al.* 1964) and a large percentage of the phospholipid in the human erythrocyte membrane (van Deenen & de Gier, 1964). The hydrolytic method may give erroneously low estimates of tissue plasmalogens in some cases (Rapport & Norton, 1962), and it would be desirable to confirm these results with a method that is not dependent on acid-lability.

Cholesterol was the major neutral lipid found in the microvillus membranes (Table 5). No esterified cholesterol was identified, in confirmation of the findings of David, Malathi & Ganguly (1966) and Ashworth & Green (1966). Though most of the cholesterol in plasma membranes of the rat appears to be unesterified, traces of cholesterol ester have been reported in the erythrocyte membrane (van Deenen & de Gier, 1964), myelin (Young & Hulcher, 1966) and the liver plasma membrane (Emmelot, Bos, Benedetti & Rumke, 1964). In the microvillus membrane almost 30% of the neutral lipid fraction was composed of fatty acids and glycerides (Table 5). The largest glyceride component was diglyceride, but there were also small amounts of mono- and tri-glyceride. Millington & Finean (1963) obtained qualitatively similar results in their thin-layer-chromatographic separation of brush-border neutral lipids. However, they were unable to demonstrate the presence of triglyceride.

An unexpected finding in the present study was the discovery that glycolipids constitute a major portion of the total membrane lipid. In Table 10 a comparison is made of the estimated neutral lipids, phospholipids and glycolipids of four rat plasma membranes. Of the four membranes the microvillus membrane possesses the greatest amount of glycolipid and the least phospholipid. These results also illustrate that the variability in the content of membrane glycolipid and phospholipid is much greater than that of neutral lipid and suggest that the more polar fractions may vary reciprocally. Recent data presented by Nelson (1967) suggest that this may also be true of erythrocyte lipids from various mammalian species, since the neutral-lipid content was extremely constant whereas reciprocal variation was noted in glycolipid and phospholipid content. It is possible that these gross variations in lipid content may reflect one manner by which specific functional characteristics are imparted to a particular membrane.

Microvilli of many species are covered by a filamentous surface coat that reacts intensely with the periodate-Schiff reagent, and also with a number of stains that are relatively specific for acid mucopolysaccharides (Ito, 1965). Though this surface layer is not well developed in the rat, electron-microscopic examination of the membranes assayed in this study (Forstner *et al.* 1968) indicates that they contain filamentous projections radiating from their external surface. These projections may be rich in carbohydrate, and it is possible that they contain much of the glycolipid found in the preparation.

It has been suggested that carbohydrate-rich coats in some membranes may affect cell permeability, control immunological reactions with the cell environment (Rambourg *et al.* 1966) and control

cell growth (Hakomori, Koscielak, Bloch & Jeanloz, 1967). Though some of these suggestions were made with regard to cell-surface glycoprotein, similar roles might be shared by some membrane glycolipids.

The fatty acid components of liver plasma membrane (Skipski *et al.* 1965) and the erythrocyte membrane (Kögl, de Gier, Mulder & van Deenen, 1960) have been reported to be predominantly saturated, whereas cellular lipids in liver (Skipski *et al.* 1965) contain a large proportion of unsaturated fatty acids. This relationship does not seem to hold for the intestine, since more than 50% of the fatty acids in the polar or major lipid fraction of the microvillus membrane were unsaturated and the fatty acid profiles of membrane and cellular lipids were quite similar (Table 8). Both membrane and cellular polar lipids increased their content of linoleic acid after safflower oil was given, indicating that their fatty acid composition could be altered readily by dietary change. Though membrane polar lipids incorporated less linoleic acid than cellular polar lipids, they incorporated more oleic acid, so that the relative proportions of saturated and unsaturated fatty acids in the membrane and cell remained similar.

The fatty acid composition of the glycerides of plasma membranes appears not to have been previously determined. Glycerides have been found in the erythrocyte (Mead & Fillerup, 1957) and liver plasma membranes (Skipski *et al.* 1965), but their significance is unknown. Analysis of the separated diglyceride and triglyceride fractions was undertaken to determine whether there was a distinctive pattern in the microvillus membrane compared with the rest of the cell. Membrane diglycerides differed markedly from cellular diglycerides in their saturated fatty acid content and relative resistance to linoleic acid incorporation (Tables 8 and 9). In addition, minor differences between membrane and cellular triglycerides became apparent in rats given safflower oil. It is clear that the distinctive fatty acid pattern in membrane diglycerides could not simply be the result of non-specific adsorption of the cellular lipid on the microvillus membrane during tissue fractionation. Therefore it is possible that the plasma membrane may contain specific glycerides that may be important in membrane function or structure.

This work was supported in part by grants from the John A. Hartford Foundation Inc. and the National Institutes of Health (AM-01392 and 03014).

REFERENCES

- Abramson, D. & Blecher, M. (1964). *J. Lipid Res.* **5**, 628.
 Ashworth, L. A. E. & Green, C. (1966). *Science*, **151**, 210.
 Autilio, L. A., Norton, W. T. & Terry, R. D. (1964). *J. Neurochem.* **11**, 17.
 Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
 Börgström, B. (1952). *Acta physiol. scand.* **25**, 101.
 Coleman, R. & Finean, J. B. (1966). *Biochim. biophys. Acta*, **125**, 197.
 David, J. S. K., Malathi, P. & Ganguly, J. (1966). *Biochem. J.* **98**, 662.
 Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.
 de Gier, J. & van Deenen, L. L. M. (1961). *Biochem. J.* **106**, 381.
 Dittmer, J. C. & Lester, R. L. (1964). *J. Lipid Res.* **5**, 126.
 Dole, V. P. (1956). *J. clin. Invest.* **35**, 150.
 Eichholz, A. (1967). *Biochim. biophys. Acta*, **135**, 475.
 Eichholz, A. & Crane, R. K. (1965). *J. Cell Biol.* **26**, 687.
 Emmelot, P., Bos, C. J., Benedetti, R. L. & Rumke, P. H. (1964). *Biochim. biophys. Acta*, **90**, 126.
 Fast, P. G. (1967). *Science*, **155**, 1680.
 Finean, J. B. (1953). *Experientia*, **9**, 17.
 Finean, J. B., Coleman, R. & Green, W. A. (1966). *Ann. N.Y. Acad. Sci.* **137**, 414.
 Folch, J., Lees, M. & Sloane-Stanley, G. H. (1956). *J. biol. Chem.* **226**, 497.
 Forstner, G., Sabesin, S. & Isselbacher, K. J. (1968). *Biochem. J.* **106**, 381.
 Hakomori, S., Koscielak, J., Bloch, K. J. & Jeanloz, R. W. (1967). *J. Immunol.* **98**, 31.
 Harris, G. & MacWilliams, I. C. (1954). *Chem. & Ind.* p. 279.
 Heimberg, M., Weinstein, I., Dishmon, G. & Fried, M. (1965). *Amer. J. Physiol.* **209**, 1053.
 Hoffman, J. F., Schulman, J. H. & Eden, M. (1959). *Fed. Proc.* **18**, 70.
 Ito, S. (1965). *J. Cell Biol.* **27**, 475.
 Johnson, C. F. (1967). *Science*, **155**, 1670.
 Kinter, W. B. & Wilson, T. H. (1965). *J. Cell Biol.* **25**, 19.
 Kögl, F., de Gier, J., Mulder, I. & van Deenen, L. L. M. (1960). *Biochim. biophys. Acta*, **43**, 95.
 Lipsky, S. R. & Landowne, R. A. (1963). In *Methods in Enzymology*, vol. 6, p. 513. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Marcus, A. J., Ullman, A. L. & Wolfman, M. (1960). *J. Lipid Res.* **1**, 179.
 Marinetti, G. V. (1962). *J. Lipid Res.* **3**, 1.
 Mead, J. F. & Fillerup, D. L. (1957). *J. biol. Chem.* **227**, 1009.
 Millington, P. F. & Finean, J. B. (1962). *J. Cell Biol.* **14**, 125.
 Millington, P. F. & Finean, J. B. (1963). In *Biochemical Problems of Lipids*, p. 125. Ed. by Frazer, A. C. Amsterdam: Elsevier Publishing Co.
 Nelson, G. J. (1967). *Biochim. biophys. Acta*, **144**, 221.
 Nussbaum, J. L., Bieth, R. & Mandel, P. (1963). *Nature, Lond.*, **198**, 586.
 Rambourg, A., Neutra, M. & Leblond, C. P. (1966). *Anat. Rec.* **154**, 41.
 Rapport, M. M. & Norton, W. T. (1962). *Annu. Rev. Biochem.* **31**, 103.
 Skidmore, W. D. & Entenman, C. (1962). *J. Lipid Res.* **3**, 471.
 Skipski, V. P., Barclay, M., Archibald, F. M., Terebus-Kekish, E., Reichman, E. S. & Good, J. J. (1965). *Life Sci.* **4**, 1673.

- van Deenen, L. L. M. & de Gier, J. (1964). In *The Red Blood Cell*, p. 252. Ed. by Bishop, C. & Surgenor, D. M. New York: Academic Press Inc.
- van Deenen, L. L. M., Houtsmuller, U. M. T., de Haas, G. H. & Mulder, E. (1962). *J. Pharm., Lond.*, **14**, 429.
- Van Handel, E. & Zilversmit, D. B. (1957). *J. Lab. clin. Med.* **50**, 152.
- Webster, G. R. (1960). *Biachim. biophys. Acta*, **44**, 109.
- Wells, M. A. & Dittmer, J. C. (1963). *Biochemistry*, **2**, 1259.
- Wherrett, J. R. (1967). *Clin. chim. Acta*, **16**, 135.
- Young, F. & Hulcher, F. H. (1966). *Proc. Soc. exp. Biol., N.Y.*, **123**, 385.
- Zlatkis, A., Zak, B. & Boyle, A. J. (1953). *J. Lab. clin. Med.* **41**, 486.