ACYLATION OF LYSOPHOSPHATIDES BY PLASMA MEMBRANE FRACTIONS OF RAT LIVER

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ABSTRACT

The plasma membrane fraction of rat liver was isolated and incubated with labeled lysophosphatides in the presence of cofactors; the acylation of lysolecithin to lecithin by the fraction was compared to that of the rough and smooth microsomes. The purity of the isolated fractions was ascertained by enzyme markers and electron microscopy, and the maximal contamination of the plasma membrane fraction by microsomes did not exceed 20%. Under conditions at which the reaction was proportional to the amount of enzyme used, the plasma membrane had a specific activity similar to that of the smooth and rough microsomes. With doubly labeled lysolecithin (containing palmitic acid-^{14}C and choline-^{3}H) it was shown that the lecithin formed retained the same ratio of the two labels, which indicated that lysolecithin was converted to lecithin through an acylation reaction. The newly formed lecithin was shown to be bound to the plasma membrane fraction; this suggested that it is incorporated into the structure of the membrane itself.

INTRODUCTION

In recent years, interest has been focused on plasma lysolecithin as the precursor of cellular lecithin (1–4). This lysophospholipid was shown to be acylated extensively by the liver (5, 6) and murine placenta (6) in vivo; considerable acylating activity was also found in arterial tissues (7, 8). In addition, it has been found that the rate of turnover of the total phospholipids of the plasma membrane of rat liver is essentially the same as that of the endoplasmic reticulum (9, 10).

It was therefore of interest to determine whether acylating enzymes, known to be localized in microsomes (11), are situated also in the plasma membrane; their presence would enable a direct utilization of circulating lysolecithin for plasma membrane lecithin formation. Three different enzymes active in lipid metabolism have been described in the plasma membrane of rat liver: a heparin-activated lipase (12), an inositol kinase (13), and a long-chain acyl CoA synthetase (14). The present paper deals with an investigation of lecithin synthesis by purified plasma membrane preparations from rat liver.

MATERIAL AND METHODS

Male albino rats of the Sprague-Dawley strain, which had been fed Purina Rat-Mouse Chow and weighed 150–200 g, were used.

Cell Fractionation

All operations were carried out at 0°–4°. Livers from two to three rats were minced with scissors, passed through a tissue press, and homogenized in 5 volumes of 0.25 M sucrose by means of 70 strokes in a hand homogenizer with a rubber pestle (15). The homogenate was then filtered through 110-mesh nylon bolting cloth.

Isolation of Plasma Membranes

The homogenate was centrifuged at 1,000 *g* for 10 min to yield a pellet containing plasma membranes, nuclei, mitochondria, and erythrocytes. This pellet

was suspended in a small volume (~1 ml/g liver) of 0.25 M sucrose–1 mM $MgCl_2$, by means of two or three strokes by hand in a ground-glass homogenizer with a loose fitting pestle. A calculated volume of 2.45 M sucrose–1 mM $MgCl_2$ was added, so that the final sucrose concentration was 1.60 M; and 5 ml of 0.25 M sucrose was layered over 30 ml of the suspension in a Spinco rotor 30 tube. Centrifugation at 30,000 rpm for 60 min yielded a plug at the interface which consisted of plasma membranes with a number of contaminating mitochondria.

The plug was washed once with 0.25 M sucrose–1 mM $MgCl_2$ (~10 ml/g liver) and once with 0.25 M sucrose–1 mM EDTA adjusted to pH 7.5. For both washes, the material was suspended by hand with a ground-glass homogenizer, and the membranes were obtained by centrifugation at 1,000 *g* for 10 min.

With the same technique described above, the material was suspended in 1.45 M sucrose–1 mM EDTA adjusted to pH 7.5, overlayered with 0.25 M sucrose, and plasma membranes which were free of the majority of contaminating mitochondria were obtained as a plug at the interface after centrifugation for 45 min at 40,000 rpm in the Spinco 40 rotor. After washing the membranes once with 0.25 M sucrose–1 mm EDTA adjusted to pH 7.5, plasma membranes were obtained by reflotation from 1.35 M sucrose–1 mM EDTA after centrifugation for 45 min at 40,000 rpm in the Spinco 40 rotor. In our hands, this procedure resulted in a reproducible higher yield of plasma membranes (~1 mg protein/g liver) than the flotation technique described by Emmelot et al. (16).

Other Subcellular Fractions

The supernate from the 1,000 *g* centrifugation was centrifuged at 10,000 *g* for 10 min, and the ensuing supernate was used for the preparation of either total microsomes (9) or smooth and rough microsomes (17).

The mitochondrial fraction used in these experiments was obtained from the pellet after centrifugation in 1.45 M sucrose–1 mM EDTA as described above. The material was suspended in 0.25 M sucrose, and, after a preliminary centrifugation at 1,000 *g* for 10 min, the mitochondria were recovered by centrifugation at 10,000 *g* for 10 min.

Labeled Substrates

Rat liver lecithin was labeled biosynthetically with either 1-^{14}C palmitic acid (5) or with choline-^{3}H (18) and was converted to lysolecithin (1-acyl-Sn-glycero-3-phosphoryl choline) by using *Crotalus adamanteus* venom (19). Lysophosphatidyl ethanolamine (1-acyl-Sn-glycero-3-phosphoryle thanolamine) was isolated from rat liver (5) after injection of ethanolamine-^{3}H.

The lysophosphatides were homogenized in water in an all-glass conical homogenizer prior to addition to the incubation mixture. Labeled lecithin prepared as above was suspended in rat serum by homogenization in an all-glass conical homogenizer.

Enzyme Assays

Aliquots of preparations of plasma membrane, microsomes, and mitochondria were incubated with either labeled lysolecithin, or L-α-glycero-phosphate-UL-^{14}C in the presence of ATP, $MgCl_2$, CoA, sodium-oleate, and phosphate buffer at 37° in a shaking incubator. The composition of the media is described in the tables. The reaction was stopped by the addition of 20 volumes of chloroform:methanol 2:1 (v/v) to the incubation mixture.

Glucose - 6 - phosphatase, 5′ - nucleotidase, cytochrome oxidase, and NADH-cytochrome *c* reductase were determined by procedures described elsewhere (16, 17, 20).

Analytical Procedures

Lipids were extracted with chloroform:methanol 2:1 (v/v) and were purified according to Folch et al. (21). The total lipids were adsorbed on silicic acid columns (22); neutral lipids were eluted with chloroform and phospholipids with methanol, containing 3% water. Neutral lipids and phospholipids were fractionated by thin-layer chromatography (23). The components, identified with the help of reference standards, were scraped off the plate into counting vials containing 0.5 ml methanol. Scintillation fluid (24) was added, and the samples were counted in the Tri Carb scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). In experiments in which lysolecithin labeled with both ^{14}C and ^{3}H was used, the fractions containing lysolecithin and lecithin were extracted according to Goldrick and Hirsch (25) with two times 10 ml of methanol (containing 3% water), and the methanolic extract was evaporated and counted.

Protein was determined according to Lowry et al. (26) and lipid phosphorus according to Bartlett (27).

Sample pellets of plasma membrane were fixed in phosphate-buffered 1% osmium tetroxide (pH 7.4), stained in block with 0.5% uranyl acetate in acetate-Veronal buffer, dehydrated in graded ethanols, and embedded in Epon (28). Sections prepared with a diamond knife on a Servall M II ultramicrotome were stained with uranyl acetate and with lead citrate (29) and were examined with a Siemens electron microscope at 60 kv.

Choline methyl-^{3}H chloride and palmitic acid-1-^{14}C were obtained from Nuclear-Chicago Corporation, Chicago, Ill.; L-α-glycerol phosphate-UL-^{14}C was obtained from International Chemical and Nuclear Corporation, City of Industry, Calif.

RESULTS

A typical analysis of the activity of marker enzymes in the subcellular fractions used in these experiments is shown in Table I. The maximum contamination of the plasma membrane fraction by mitochondria was ~5%, as judged by cytochrome oxidase activity, and contamination by elements of the endoplasmic reticulum ranged between 10 and 20%, as determined by NADH-cytochrome *c* reductase or glucose-6-phosphatase activity. A more detailed characterization of the plasma membrane fraction will be presented elsewhere.

TABLE I

Activity of Marker Enzymes in the Fractions Studied

Fraction	Specific activities			
	6-glucose-phosphatase*	5′-nucleotidase‡	NADH-cytochrome *c* reductase§	Cytochrome‖ oxidase
	μmoles	*μmoles*	*mμmoles*	*mμmoles*
Homogenate	1.40	1.36	158	175
Mitochondria	0.27	0.63	128	477
Microsomes	4.28	2.78	342	17
Plasma membrane	0.85	20.40	390	21

* P_i liberated from Glucose-6-phosphate per 20 min per milligram protein at 37°.

‡ P_i liberated from 5′ AMP per 20 min per milligram protein at 37°.

§ Cytochrome *c* reduced per minute per milligram protein at room temperature.

‖ Cytochrome *c* oxidized per minute per milligram protein at room temperature.

When they were incubated with labeled lysolecithin and cofactors, the subcellular fractions studied showed acylating activity as indicated by the formation of labeled lecithin. When expressed as lecithin formed per milligram protein in the fraction, the specific activity of the plasma membrane preparations was comparable to that displayed by the whole microsomal fraction. The enzymatic activity in the plasma membrane and microsomes was proportional to the amount of protein present in this concentration range (Table II); however, high concentrations of plasma membrane protein had an inhibitory effect. As seen in Table II, the specific activity of the enzyme in both preparations was the same at different protein concentrations. These findings indicate that the acylating activity found in the plasma membrane fraction is not due to microsomes of which the maximal contamination was estimated at 20%. The specific activity of the acylating enzyme in the mitochondrial fraction was lower than that in the microsome or plasma membrane fraction, and there was only negligible activity in the particle-free supernatant. When ATP and CoA were omitted from the incubation mixture, only minimal conversion of lysolecithin to lecithin occurred. The plasma membrane acylating activity was further compared to the activity of rough and smooth microsomes, and as seen in Table III, all preparations show comparable activities. Since

TABLE II

Conversion of Lysolecithin to Lecithin by Plasma Membrane Fraction of Rat Liver

Experiment	Fraction		Lecithin formed
		mg protein	*mμmoles/mg protein*
1	Plasma membrane	0.88	33.0
	Microsomes	0.70	48.0
2	Plasma membrane	0.20	49.0
		0.40	41.0
		0.40 ATP α CoA omitted	2.7
	Microsomes	0.14	40.0
		0.70	44.0
	Mitochondria	0.72	9.0

The incubation mixture contained 0.05 mM 1-acyl-Sn-glycero-3-phosphorylcholine-^{3}H, 5 mM K-ATP, 0.05 mg CoA, 0.05 mM sodium oleate, 0.02 M K-phosphate buffer (pH 7.4), and 5 mM $MgCl_2$, which was made up to a final volume of 1.0 ml with 0.15 M KCl–0.5 M Tris buffer pH 7.4 (19:1).

TABLE III

Conversion of Monoacyl to Diacylphosphatides by Plasma Membrane and Microsome Fractions of Rat Liver

Fraction		Lecithin formed from lysolecithin	Phosphatidyl ethanolamine formed from lysophosphatidyl ethanolamine
	mg protein	*mμmoles/mg protein*	
Plasma membrane	0.20	76.1	62.0
Rough microsomes	0.16	79.2	—
	0.80	69.6	—
Smooth microsomes	0.16	77.0	—
	0.80	68.3	—

Conditions of incubation as in Table II; the concentration of 1-acyl-Sn-glycero-3-phosphoryl ethanolamine-^{3}H was 0.05 mM.

the separation of rough from smooth microsomes required the presence of CsCl, the activity of the respective fractions was compared to that of whole microsomes, and no inhibitory effect of CsCl was found. The plasma membrane preparation was also shown to acylate lysophosphatidyl ethanolamine to phosphatidyl ethanolamine (Table III).

The mode of conversion of lysolecithin to lecithin was studied by using lysolecithin labeled in the 1-position with palmitic acid-1-^{14}C and with choline-^{3}H. The ^{14}C to ^{3}H ratio of the newly synthesized lecithin was compared to that of the lysolecithin in the reaction mixture before and after incubation, and it was found to be similar (Table IV).

It seemed of interest to determine whether newly synthesized lecithin is loosely bound to the plasma membrane and easily exchangeable with lecithin in the incubation medium. The experiment shown in Fig. 1 was designed to test this possibility. After incubation the mixture was centrifuged, and 77% of the total radioactivity, which contained all the newly formed lecithin, sedimented with the plasma membrane (tube *A*). After resuspension of the membranes in serum and sedimentation through 1.2 M sucrose, 65% of this radioactivity remained with the membranes; of this, 83% was lecithin, and only 5% of the newly formed lecithin was recovered in the serum. In the control tube *C*, only 9% of the added lecithin sedimented through 1.2 M sucrose with the membranes.

In order to learn whether the newly synthesized lecithin forms an integral part of the membrane or whether it is deposited in the form of myelin figures, the preparations incubated with lysolecithin were checked by electron microscopy. In numerous sections examined, no myelin figure formation was observed. The plasma membrane preserved its structural integrity, and in many regions junctional elements were apparent (Figs. 2 and 3).

The presence of other enzymes active in lipid synthesis in the plasma membrane was also studied by using L-α-glycero-^{14}C phosphate as an acyl acceptor. As seen in Table V, addition of oleic acid

TABLE IV

The ^{14}C/^{3}H Ratio in Lecithin Formed from 1-Palmitoyl-1-^{14}C-Sn-Glycero-3-Phosphorylcholine-^{3}H by Plasma Membrane Fraction of Rat Liver

	^{14}C/^{3}H Ratio	
	Lysolecithin	Lecithin
Reaction mixture		
Before incubation	1.00	—
After incubation	1.00	1.12

Conditions of incubation as in Table II; the concentration of sodium oleate was 0.1 mM.

to the incubation mixture enhanced the incorporation of α-glycerophosphate into total lipids. At optimal fatty acid concentration, the activity of the microsomal fraction exceeded that of both the plasma membrane and mitochondrial preparations. However, when the protein concentration of the microsomes and mitochondria was reduced to about 20% of the plasma membrane protein concentration (maximal contamination by microsomes), the rate of α-glycerophosphate esterification was reduced drastically to less than 1 mμmole/mg protein. Since this reaction is not linear at low protein concentration (less than 500 μg/ml) (30), it was not possible to use this type of dilution method to ascertain whether the activity in the plasma membrane might be due to contamination with microsomes.

This enzyme activity of the plasma membrane preparation was also compared with that found in isolated fractions of rough and smooth endoplasmic

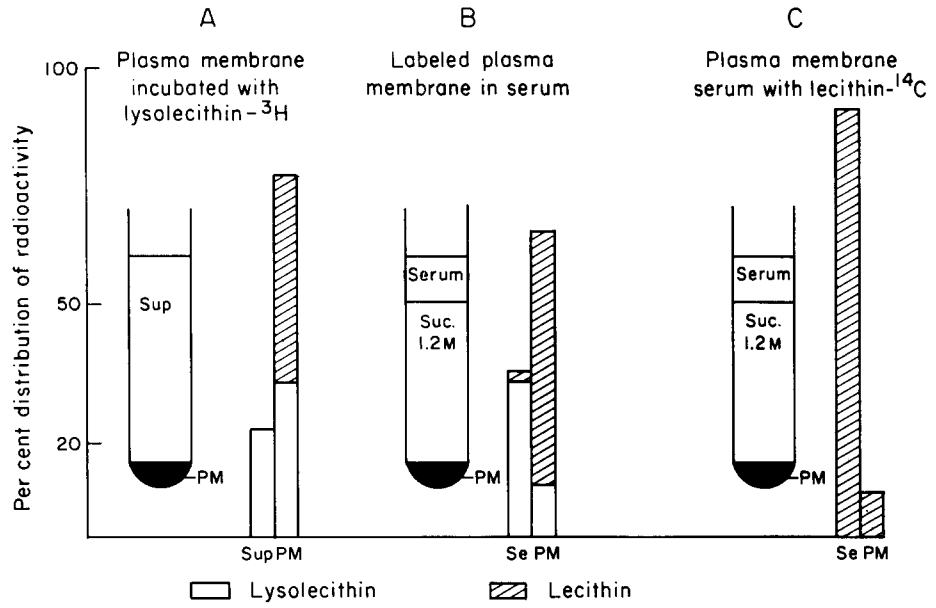

FIGURE 1 The mixture, containing plasma membrane (1 mg protein), 0.05 mM lysolecithin, 5 mM ATP, 0.25 mg CoA, 0.05 mM sodium oleate, 5 mM $MgCl_2$, 0.02 M K-phosphate buffer (pH 7.4), made up to a final volume of 5.0 ml with KCl-tris buffer, was incubated for 30 min at 37°. It was then centrifuged at 12,000 *g* for 10 min to sediment the labeled plasma membrane (*PM*; tube *A*). A duplicate pellet was resuspended in 1.5 ml rat serum and layered over 1.2 M sucrose (*Suc*; tube *B*). Another pellet of plasma membrane obtained after incubation in an unlabeled medium was resuspended in rat serum which contained labeled rat liver lecithin (tube *C*). Tubes *B* and *C* were then centrifuged in a SW 39 swinging bucket for 4.5×10^6 *g*-min, and labeled lipids were analyzed in the serum, sucrose, and sedimented plasma membrane. The per cent distributions of the radioactivity in serum and plasma membrane are shown in the bar diagrams; the radioactivity found in the sucrose was negligible and has been added to that of the serum. *Sup*, supernatant; *Se*, serum.

reticulum, both of which showed a higher activity. The lipid formed by the various preparations was shown to be predominantly phosphatidic acid. In the presence of postmicrosomal supernate, the formation of neutral glycerides, mainly di- and triglycerides, was increased (from 5 to 20% of the incorporated radioactivity), and about 20% of the radioactivity found in the phospholipid fraction was in lecithin.

DISCUSSION

The present study provides evidence that the plasma membrane fraction isolated from rat liver contains an enzyme active in the conversion of lysolecithin to lecithin. Since the newly formed lecithin is not released when plasma membranes are suspended in serum and does not exchange with lecithin in the serum, we assume that the newly synthesized phosphatide forms an integral part of the membrane.

The finding of equal specific activity in both the plasma membrane and microsomes precluded the possibility that the contamination with the latter could account for the enzymic activity found in the plasma membrane, since enzymic activity was proportional to the protein concentration in the range used. Lysolecithin was converted to lecithin to a similar extent by both the rough and the smooth microsomes. The rate of conversion was lower in mitochondria. The ATP and CoA dependence of the reaction and the conservation of the $^{14}C/^{3}H$ ratio in the lecithin which was derived from palmitoyl-^{14}C and choline-^{3}H lysolecithin indicated that formation of lecithin proceeds through the acylation pathway and not through the condensation of two lysolecithin molecules. Similar results were obtained in a previous study in which a conservation of the $^{14}C/^{3}H$ ratio in whole liver lecithin was observed following the intravenous injection of doubly labeled lysolecithin

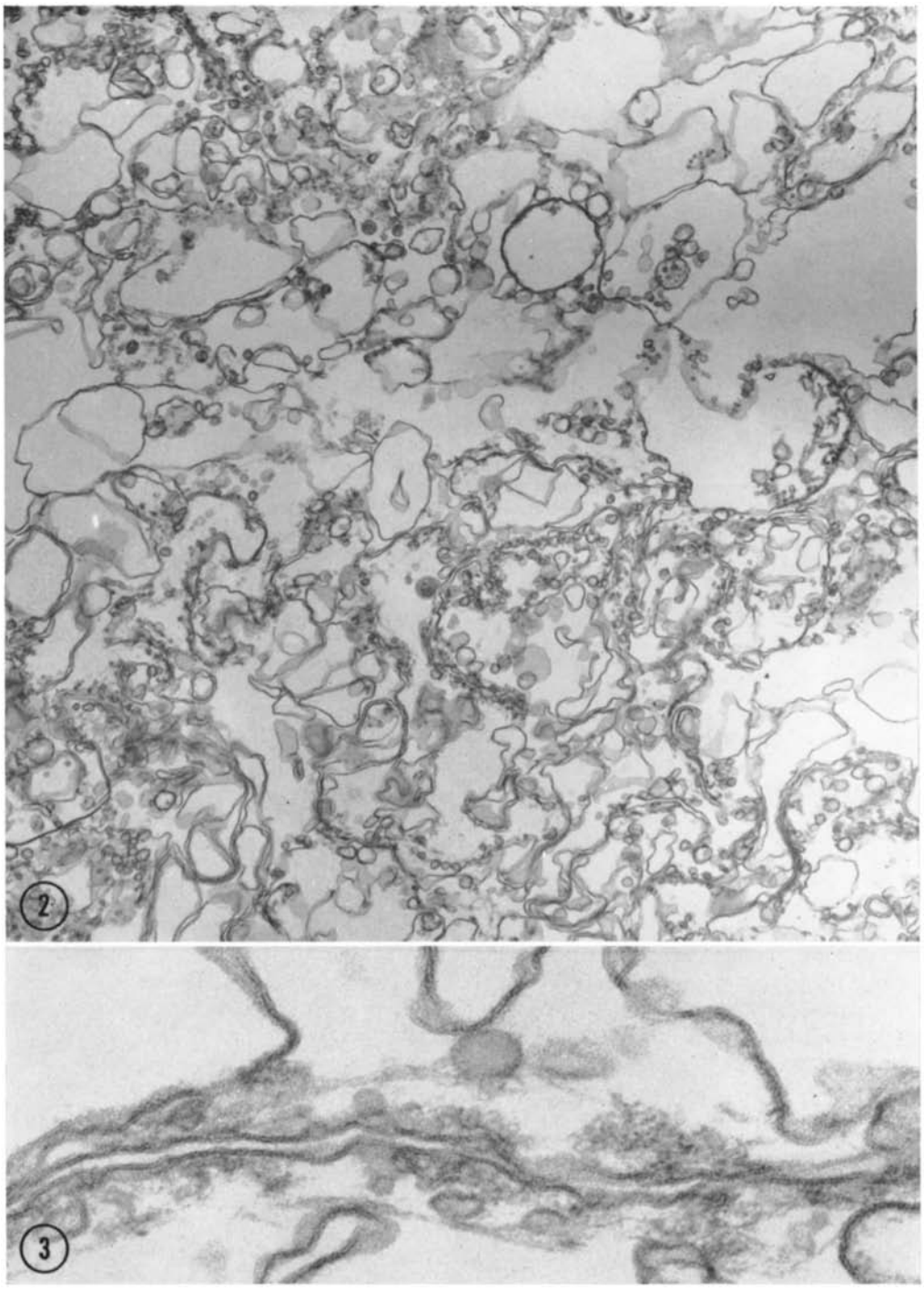

FIGURE 2 Section of plasma membrane preparation after incubation with lysolecithin. × 10,000.
FIGURE 3 Portion of plasma membrane after incubation with lysolecithin, showing preservation of unit membrane structure. × 105,000.

TABLE V

Incorporation of L-α-*glycerophosphate-UL-*^{14}C *into Esterified Lipids by Plasma Membrane of Rat Liver*

Fraction		Additions	L-α-glycerophosphate esterified into lipid
	mg protein		*mμmoles/mg protein*
Plasma membrane	1.12	None	2.7
	1.12	Sodium oleate 0.05 mM	4.5
	1.12	Sodium oleate 0.2 mM	10.3
Microsomes	1.12	Sodium oleate 0.2 mM	38.1
	0.24	Sodium oleate 0.2 mM	<1.0
Mitochondria	1.22	Sodium oleate 0.2 mM	7.0
	0.24	Sodium oleate 0.2 mM	<1.0

Conditions of incubation as in Table II; L-α-glycerophosphate-UL-^{14}C, 2.5 mM served as substrate.

(5). The finding of the high acylating activity in plasma membrane preparations raised the question of how extensive this enzymic activity is in vivo. Labeled lecithin was found in the plasma membrane of liver 5 min after intravenous injection of labeled lysolecithin, and its specific activity was only 30% less than that of the smooth or rough microsomes. The finding that the plasma membrane is active in acylating lysolecithin to lecithin is relevant to recent studies on the turnover of cell membrane components. These studies (9, 10) have shown that the protein and phospholipid components of both endoplasmic reticulum and plasma membrane turnover at different rates, the glycerophospholipids having a shorter half-life than the proteins. These findings were discussed in respect to the different concepts of membrane assembly, and it was pointed out that the difference in turnover rates does not support the hypothesis of assembly from preformed lipoprotein subunits of uniform composition. It follows that the rapid turnover of membrane glycerophospholipids, of which more than 50% are accounted for by lecithin and phosphatidyl ethanolamine, requires an active regenerating system. Hence, the presence of enzymes acylating lysolecithins and lysophosphatidyl ethanolamine in the plasma membrane is functionally justified, and it is plausible that these enzymes participate in the renewal of both membrane lecithin and membrane phosphatidyl ethanolamine *in situ*.

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REFERENCES

1. Oliveira, M. M., and M. Vaughan. 1962. *Federation Proc.* **21**:296.
2. Van Deenen, L. L. M., J. De Gier, U. M. T. Houtsmuller, A. Montfoort, and E. Mulder. 1953. *In* Biochemical Problems of Lipids. A. C. Frazer, editor. Elsevier Publishing Co., Amsterdam, the Netherlands. 404.
3. Polonovski, J., and M. Paysant. 1963. *Bull. Soc. Chim. Biol.* **45**:339.
4. Elsbach, P., J. W. O. Van den Berg, H. Van den Bosch, and L. L. M. Van Deenen. 1965. *Biochim. Biophhys. Acta.* **106**:338.
5. Stein, Y., and O. Stein. 1966. *Biochim. Biophys. Acta.* **116**:95.
6. Eisenberg, S., Y. Stein, and O. Stein. 1967. *Biochim. Biophys. Acta.* **137**:115.
7. Eisenberg, S., Y. Stein, and O. Stein. 1967. *Biochim. Biophys. Acta.* **137**:221.
8. Portman, O. W. 1967. *J. Atherosclerosis Res.* **7**: 617.

9. OMURA, T., P. SIEKEVITZ, and G. E. PALADE. 1967. *J. Biol. Chem.* **242**:2389.
10. WIDNELL, C. C., and P. SIEKEVITZ. 1967. *J. Cell Biol.* **35**:142A.
11. LANDS, W. E. M. 1960. *J. Biol. Chem.* **235**:2233.
12. HIGGINS, J. A., and C. GREEN. 1967. *Biochim. Biophys. Acta.* **144**:211.
13. MICHELL, R. H., J. L. HARWOOD, R. COLEMAN, and J. N. HAWTHORNE. 1967. *Biochim. Biophys. Acta.* **144**:649.
14. PANDE, S. V., and J. F. MEAD. 1968. *J. Biol. Chem.* **243**:352.
15. JACOB, S. T., and P. M. BHARGAVA. 1962. *Exptl. Cell Res.* **27**:453.
16. EMMELOT, P., C. J. BOS, E. L. BENEDETTI, and P. H. RUMKE. 1964. *Biochim. Biophys. Acta.* **90**: 126.
17. DALLNER, G., P. SIEKEVITZ, and G. E. PALADE. 1966. *J. Cell Biol.* **30**:73.
18. RACHMILEWITZ, D., S. EISENBERG, Y. STEIN, and O. STEIN. 1967. *Biochim. Biophys. Acta.* **144**: 624.
19. LONG, C., and I. F. PENNY. 1957. *Biochem. J.* **65**: 382.
20. COPPERSTEIN, S. J., and A. LAZAROW. 1951. *J. Biol. Chem.* **189**:665.
21. FOLCH, J., M. LEES, and G. H. SLOANE-STANLEY. 1957. *J. Biol. Chem.* **226**:497.
22. NEWMAN, H. A. I., C. T. LIU, and D. B. ZILVERSMIT. 1961. *J. Lipid Res.* **2**:403.
23. SKIPSKI, V. P., R. F. PETERSON, and M. BARCLAY. 1964. *Biochem. J.* **90**:374.
24. BRAY, G. A. 1960. *Anal. Biochem.* **1**: 279.
25. GOLDRICK, B., and J. HIRSCH. 1963. *J. Lipid Res.* **4**:482.
26. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDLE. 1951. *J. Biol. Chem.* **193**: 265.
27. BARTLETT, G. R. 1959. *J. Biol. Chem.* **234**:466.
28. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **11**: 736.
29. REYNOLDS, E. S. 1963. *J. Cell Biol.* **17**:208.
30. TZUR, R., and B. SHAPIRO. 1964. *J. Lipid Res.* **5**: 542.