Comparison of Mitochondrial with Microsomal Acylation of Monoacyl Phosphoglycerides*

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ABSTRACT: Monoacyl phosphoglyceride acylation catalyzed by enzymes in outer membrane preparations of rat liver mitochondria has been compared with that catalyzed by microsomal enzymes under various conditions in an attempt to demonstrate that activity in the mitochondrial preparation is the result of a mitochondrial enzyme. Specific activities of the microsomal acylase(s) were 3.5–0.8 times that of the outer membrane acylase. By contrast, the specific activity of microsomal marker enzymes was six- to tenfold higher for microsomes than for outer membrane preparations; outer membrane activity therefore cannot be ascribed to microsomal contamination. Monoacylglycerophosphorylethanolamine was a better acyl acceptor than monoacylglycerophosphorylcholine. Comparison of acylase activity with that of monoamine oxidase (an outer membrane enzyme) in mitochondrial outer and inner membranes demonstrates that most if not all of the acylase is in the outer membrane. Addition of a lysosome-rich fraction to either the outer membranes or microsomes produced a marked inhibition of the acylase; this might account for the failure of some workers to find acylase activity in crude mitochondrial preparations. The acylase(s) could utilize endogenous monoacylphosphoglycerides at rates comparable with rates found for incorporation of fatty acids.

Outer membranes incorporated most of the linoleic acid into the 2 position of endogenous phosphatidylethanolamine, whereas microsomes incorporated most linoleic acid into the 1 position.

Accumulation of monoacyl phosphoglycerides as a result of action of a mitochondrial phospholipase A was first reported in 1965 (Rossi *et al.*; Scherphof and van Deenen). This enzyme acts primarily on the 2-acyl group of exogenous phosphatidylethanolamine and therefore is categorized as a phospholipase A_2 (Scherphof *et al.*, 1966; Waite and van Deenen, 1967). Subsequent studies provided evidence that the mitochondrial phospholipase A is distinct from the lysosomal phospholipase A (Vignais and Nachbaur, 1968; Nachbaur and Vignais, 1968; Waite *et al.*, 1969a) and is one factor involved in mitochondrial swelling (Waite *et al.*, 1969b). This enzyme is mainly localized in the mitochondrial outer membrane (Nachbaur and Vignais, 1968; Waite, 1969) although some inner membrane phosphatidylethanolamine undergoes hydrolysis which cannot be ascribed to the activity of contaminating outer membrane phospholipase A.

These results raise a question concerning the fate of the monoacyl phosphoglycerides produced by the action of the mitochondrial phospholipase A. As isolated, mitochondria contain less than 2% of the phosphoglycerides as the monoacyl phosphoglycerides. On incubation with Ca^{2+}, however, up to 70% of the phosphatidylethanolamine can be converted into the monoacyl compound (Bjørnstad, 1966; Waite *et al.*, 1969a). There are at least three possible pathways by which the cell could further metabolize mitochondrial monoacyl phosphoglycerides: (1) further hydrolysis to free fatty acid and glycerophosphorylethanolamine (or -choline), (2) exchange of the phosphoglycerides into the endoplasmic reticulum where they could be further metabolized (Lands, 1960), or (3) reacylation in the mitochondrial outer membrane to give the diacyl phosphoglyceride. The first possibility does not seem to constitute a major pathway, since only 20% of the monoacyl phosphoglyceride formed by mitochondria has been found to undergo further hydrolysis (Bjørnstad, 1966; Waite and van Deenen, 1967; Waite *et al.*, 1969a). An exchange of phospholipids between mitochondria and the endoplasmic reticulum apparently does occur although it is not known whether this exchange involves only diacyl phosphoglycerides or whether it could also occur with monoacyl phosphoglycerides (Wirtz and Zilversmit, 1968; McMurray and Dawson, 1969). Webster (1962) and Scherphof and van Deenen (1966) reported that mitochondria contain an acyl-CoA–monoacyl phosphoglyceride acyl transferase (acylase) which can catalyze the reacylation of monoacyl phosphoglyceride. This activity has been attributed to mitochondrial outer membrane (Stoffel and Schiefer, 1968; Nachbaur *et al.*, 1969), but these results have been challenged (Eibl *et al.*, 1969).

It is the purpose of this report to examine further the nature of the mitochondrial outer membrane acylase and to exclude the possibility that the acyl transferase activity observed in the outer membrane is only the result of the presence of contaminating endoplasmic reticulum. We have made both qualitative and quantitative studies of the two cell-fraction preparations.

Experimental Procedures

Isolation of Liver Fractions. Mitochondria and the mitochondrial membrane fractions were prepared from rat livers

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as described earlier (Waite *et al.*, 1969a). The microsomes were isolated by centrifugation between 25,000*g* for 10 min and 100,000*g* for 60 min and then were osmotically swollen and contracted, sonicated, and centrifuged in a sucrose gradient in the same manner used to isolate the mitochondrial outer membrane. The lysosome-rich fraction sedimented between 4500*g* for 10 min and 25,000*g* for 10 min. In some experiments the rats were injected intraperitoneally with 20 μCi of [^{14}C]-ethanolamine 90 min before they were killed (Bjørnstad, 1966).

Acylation with [^{14}C]Fatty Acids. The membrane fractions (25 μg) were incubated for 3 min in a 1.0-ml reaction mixture containing 0.1 M Tris buffer (pH 7.5), 15 μM ^{14}C-labeled fatty acid, 2.5 mM ATP, 2.5 mM $MgCl_2$, 250 μM CoA, 300 μg of bovine serum albumin, and the following amounts of monoacyl phosphoglycerides: monoacylglycerophosphorylcholine, 25 μM with microsomes and 40 μM with outer membranes; monoacylglycerophosphorylethanolamine, 75 μM with microsomes and 100 μM with outer membranes when palmitic acid was used, 40 μM when linoleic acid was used. The monoacyl phosphoglycerides and fatty acids were added as ultrasonic suspension (Waite *et al.*, 1969a). Any alteration of the incubation conditions is noted in the Results section. The ATP, $MgCl_2$, CoA, and albumin solution is referred to as the ATP solution.

Hydrolysis and Reacylation of Endogenous [^{14}C]Ethanolamine-Labeled Phosphatidylethanolamine. Protein (1–2 mg) of the mitochondrial outer membrane preparation from rats that had been injected with [^{14}C]ethanolamine was incubated in 1.0 ml of a 50 mM glycylglycine buffer (pH 8.2), with 2.0 mM $CaCl_2$ for 30 min during which the [^{14}C]phosphatidylethanolamine was hydrolyzed by the membrane-bound phospholipase A. The incubation mixture was then made 3.0 mM with respect to EDTA to stop the phospholipase A activity and then the ATP solution was then added to give a final volume of 1.5 ml. The pH was adjusted to 7.4 and the incubation was continued for another 10 min unless otherwise specified. Specific activity of endogenous phosphatidylethanolamine was based on the ^{14}C and phosphorus content (Waite, 1969).

Chromatography. In those experiments in which we used ^{14}C-labeled fatty acids as substrates the products were chromatographed on the silica gel G thin-layer plates first in a chloroform–petroleum ether–acetic acid system (70:30:2, v/v) then in a chloroform–methanol–H_2O system (70:30:4, v/v). The silicic acid containing the labeled phosphoglyceride was scraped from the thin-layer chromatography plates and the radioactivity was determined in a scintillation counter. The extent of hydrolysis and reacylation of [^{14}C]ethanolamine-labeled phosphatidylethanolamine was determined in the same manner except that a chloroform–methanol–H_2O–NH_4OH system (65:35:3:4, v/v) was used.

Marker Enzymes. The methods to assay the marker were described before (Waite, 1969).

Materials. Rat liver phosphoglycerides were hydrolyzed by *Crotalus adamanteus* venom phospholipase A, which liberates the monoacyl phosphoglycerides. Linoleoyl-CoA was synthesized from linoleoyl chloride and reduced CoA (Reitz *et al.*, 1968), and 1-[9,10-^{3}H]palmitoylglycerophosphorylethanolamine was prepared as described earlier by phospholipase A hydrolysis of 1-[9,10-^{3}H]palmitoylphosphatidylethanolamine (Waite and van Deenen, 1967).

[^{14}C]Ethanolamine (specific activity 3.7 mCi/mole) and [^{3}H]oleic acid (specific activity 5 mCi/4.55 mg) were purchased from the New England Nuclear Corp., Boston, Mass. [^{14}C]-Palmitic acid (55.2 mCi/mmole), [^{14}C]oleic acid (57.2 mCi/mmole), [^{14}C]linoleic acid (52.9 mCi/mmole), and [^{14}C]glycerol (specific activity 15.4 mCi/mmole) were obtained from Amersham-Searle, Des Plaines, Ill. [^{14}C]Arachidonic acid (specific activity 52 mCi/mmole) was from Applied Science Lab, Inc., State College, Pa. Linoleoyl chloride was obtained from Analabs, Hamden, Conn. All other compounds were obtained from Sigma, St. Louis, Mo.

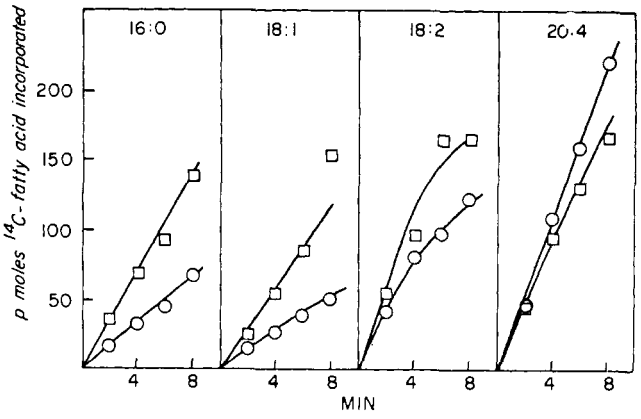

FIGURE 1: Time course of acylase assay. Microsomes (□) and outer membranes (○) (25 μg of protein) were incubated with labeled palmitic (16:0), oleic (18:1), linoleic (18:2), and arachidonic (20:4) acids, the monoacyl phosphoglyceride and the ATP solution for the indicated periods of time. The reaction products were separated by thin-layer chromatography.

Results

Acylation of Added Phosphoglycerides. Optimal conditions for microsomal and mitochondrial outer membrane acylation were determined in a variety of experiments. Figure 1 shows the rate of [^{14}C]phosphatidylethanolamine formation with palmitic (16:0), oleic (18:1), linoleic (18:2), or arachidonic (20:4) acid which was dependent upon the addition of monoacyl glycerophosphorylethanolamine. The microsomal fraction had greater activity with palmitic, oleic, and linoleic acids than the outer membrane, while arachidonic was incorporated faster by the outer membrane. Under these conditions 10–15% of the added fatty acid was incorporated and about 5% of the added monoacylglycerophosphorylethanolamine was utilized. Similar results were obtained using monoacylglycerophosphorylcholine as acyl acceptor. If the monoacyl phosphoglyceride was omitted only a small amount of [^{14}C]phosphoglyceride was formed, which appeared as a result of synthesis *de novo* or from acylation of endogenous monoacylphospholipids. The reacylation required both ATP and CoA.

Microsomes had decreased activity with small amounts (5–10 μg) of protein, which resulted in a nonlinear relation between activity and protein added (Figure 2). This was found with either monoacylglycerophosphorylethanolamine and monoacylglycerophosphorylcholine as the acyl acceptor. The nonlinear response and level of activity was not altered by repeated freezing and thawing or by sonication of the

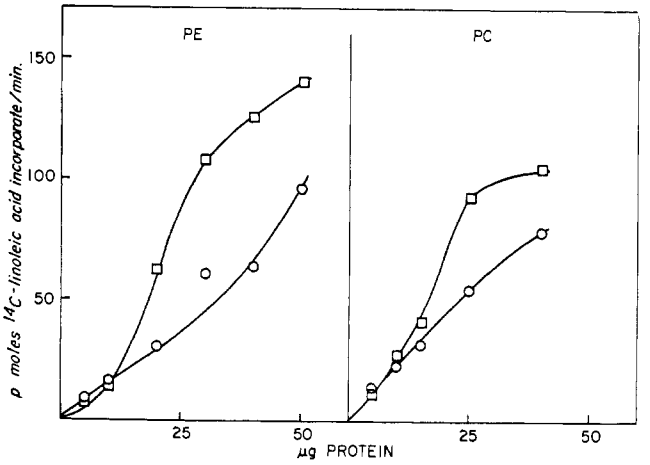

FIGURE 2: Effect of protein concentration on acylation. Various amounts of microsomes (□) and outer membranes (○) were incubated for 3 min [^{14}C]linoleic acid, monoacyl phosphoglyceride, and the ATP solution (PE = phosphatidylethanolamine, PC = phosphatidylcholine).

membrane fractions prior to addition to the incubation mixture. Any deviation from a linear response was not correlated with the presence of various amounts of outer membrane protein. These data show that the activity of the microsomes relative to that of the outer membrane varies from a ratio of 1:1 in the presence of low amounts of protein (5–10 µg) to about 2:1 in the presence of moderate amounts of protein (25 µg).

Increasing the concentration of added [^{14}C]palmitic and [^{14}C]linoleic acid to about 10 µM caused increased incorporation of the acid into both phosphatidylethanolamine and phosphatidylcholine (Figure 3). Palmitic acid was incorporated into phosphatidylethanolamine by microsomes at a rate about two-thirds that of linoleic acid. The rate of palmitic acid incorporation by outer membranes relative to that of linoleic acid was lower than that found with microsomes. Palmitic acid incorporation into phosphatidylcholine by

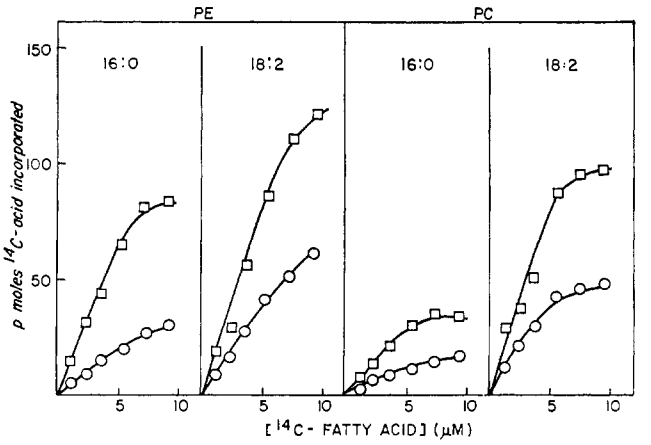

FIGURE 3: Effect of fatty acid concentration of acylase activity. Microsomes (□) and outer membrane (○) were incubated for 3 min with the indicated concentrations of the [^{14}C]fatty acids, monoacylphosphoglyceride, and the ATP solution.

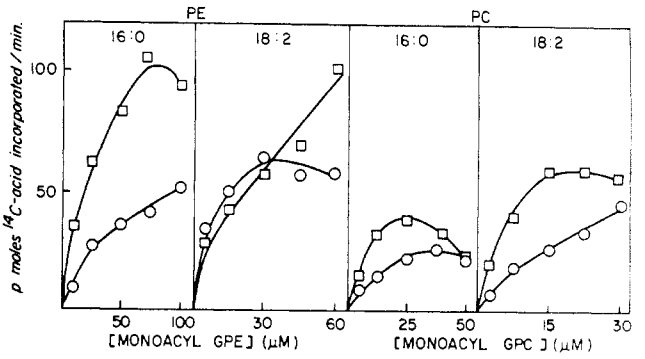

FIGURE 4: Effect of monoacyl phosphoglyceride concentration on acylation. Microsomes (□) and outer membranes (○) were incubated 3 min with the indicated concentrations of monoacylphosphoglycerides, [^{14}C]fatty acid, and the ATP solution.

both microsomes and outer membranes was low. *C. adamanteus* venom hydrolyzed 80% of the [^{14}C]palmitic acid, thus it was in the 2 position of phosphatidylethanolamine. Only 25–35% of the [^{14}C]palmitic acid was in the 2 position of the phosphatidylcholine, probably because of a detergent effect of the monoacyl phosphoglyceride stimulating synthesis *de novo* or the turnover of the phosphoglycerides at the 1 position.

The amount of added monoacyl phosphoglycerides required for saturation depended on the nature of the fatty acid and the monoacyl phosphoglyceride used (Figure 4). With palmitic acid as the substrate, the microsomal enzyme was saturated by 80 µM monoacylglycerophosphorylethanolamine and about 100 µM monoacylglycerophosphorylethanolamine saturated the outer membranes. With [^{14}C]linoleic acid as the substrate, only 30 µM monoacylglycerophosphorylethanolamine saturated the outer membrane system, and twice as much was required to saturate the microsomal systems (60 µM). With limiting monoacylglycerophosphorylethanolamine concentrations, the rate of [^{14}C]linoleate incorporation was higher with outer membranes than with microsomes. Relatively little monoacylglycerophosphorylcholine was necessary for maximal rates of acylation: 15–25 µM saturated the microsomes, with either [^{14}C]linoleic or palmitic acid as the substrate. Higher concentrations of monoacylglycerophosphorylcholine were required to saturate the outer membrane system (30–40 µM) than were necessary in the case of the microsomes. These results show that the two systems have different affinities for the substrates used.

The specific activities from seven such studies were averaged and compared with those of microsomal (TPNH–cytochrome C reductase and glucose 6-phosphatase) and outer membrane (monoamine oxidase) marker enzymes. Both of these microsomal marker enzymes were used because some question exists as to the validity of using only glucose 6-phosphatase for studying microsomal contamination in outer membrane preparations (Parsons *et al.*, 1967; Brunner and Bygrave, 1969). Specific activities of acylation varied depending on the fatty acid in the phosphoglyceride used (Table IA). The ratio of the averaged activities incorporated into phosphatidylethanolamine of microsomes to outer membranes ranged from 3.58 for palmitic acid to 0.79 for arachidonic acid; this is taken to mean that arachidonic acid is preferred

TABLE I

	Phosphatidyl-ethanolamine			Phosphatidyl-choline		
	16:0	18:2	20:4	16:0	18:2	20:4
A. Distribution of Acylases in Mitochondrial Outer Membranes and Microsomes[a]						
Outer membranes (OM)	0.62	1.89	2.75	0.26	1.50	2.0
Microsomes (M)	2.22	3.16	2.18	0.58	1.89	1.59
Ratio (M:OM)	3.58	1.67	0.79	2.2	1.26	0.80

	TPNH-Cytochrome C Reductase	Glucose 6-Phos-phatase	Monoamine Oxidase
B. Distribution of Marker Enzyme in Mitochondrial Outer Membranes and Microsomes[b]			
Outer membranes (OM)	11	15	78
Microsomes (M)	112	90	3
Ratio (M:OM)	10	6	0.04

[a] The outer membrane and microsome specific activities were determined with monoacylglycerophosphorylethanolamine and monoacylglycerophosphorylcholine and palmitic (16:0), linoleic (18:2), or arachidonic (20:4), acids in incubations run 3 min with 25 μg of protein and the ATP solution. In nanomoles per minute per milligram of protein. [b] The marker enzymes activities were determined with the same preparations used for acylation studies. In nanomoles per minute per milligram of protein.

by the outer membrane, palmitic acid by microsomes. Also, the rate of palmitic acid incorporation into phosphatidylethanolamine by microsomes is greater than into phosphatidylcholine specific activity 2.22 *vs.* 0.58). In all experiments monoacylglycerophosphorylethanolamine was a better acyl acceptor than monoacylglycerophorylcholine, as was also found to be the case by Nachbaur *et al.* (1969). The results varied among preparations. For example, the specific activities ratios of [^{14}C]linoleic acid incorporation into phosphatidylethanolamine varied from 1.20 to 2.10, a variation that could not be explained on the basis of microsomal contamination in the outer membrane fraction since the distribution of microsomal marker enzymes between the two fractions was always six to ten times as high as the ratio of acylation activities. The variation in acylase activity was not paralleled by changes in marker enzyme activities.

In Figure 5 the acylation activity of mitochondria, outer membranes, and microsomes is plotted as a function of the TPNH–cytochrome C reductase activity of the preparations. This plot (McMurray and Dawson, 1969) is another method of determining if particular enzyme activity is the result of contamination of the mitochondria preparation by microsomes; if the acylation activity were in fact due to microsomal contamination, the curves for the mitochondria, outer membranes, and microsomes should be superimposable.

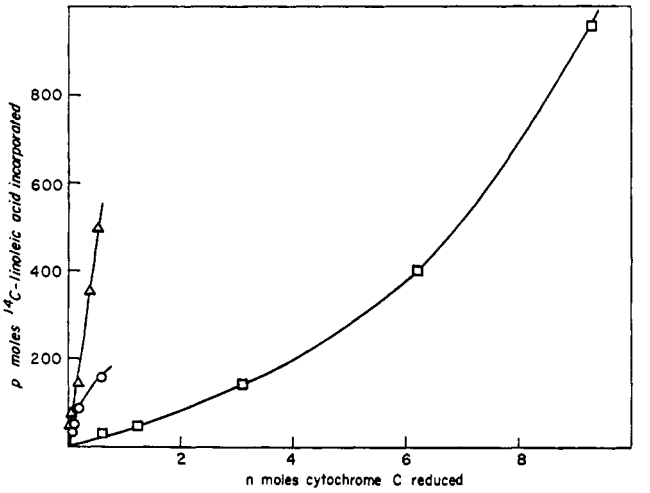

FIGURE 5: [^{14}C]Phosphatidylethanolamine synthesis and TPNH cytochrome reductase C activity in mitochondria, outer membranes, and microsome. The TPNH–cytochrome C reductase was determined as cited earlier (Waite, 1969). The acylase activity was determined as described earlier except 20–80 μg of mitochondrial protein (○), 5–75 μg of outer membrane protein (△), and 5–75 μg of microsome protein (□) were used. Each point is plotted as the amount of acylase activity *vs.* the amount of reductase activity present in the membrane preparation used.

Figure 5 shows that outer membranes and mitochondria have higher acylase than TPNH–cytochrome C reductase activity relative to the microsomes, and demonstrates that the formation of the diacyl phosphoglyceride from monoacyl phosphoglyceride by mitochondria is the result of a mitochondrial enzyme, not of microsomal contamination.

TABLE II

	Acylase (pmoles/min mg)	Monoamine Oxidase (nmoles/min mg)
A. Acylase and Monoamine Oxidase Activities in Mitochondrial Fractions[a]		
Mitochondria	200	6.2
Outer membranes	1430	46.3
Inner membranes	93	1.3
Soluble matrix	142	5.8
B. Ratio of Specific Activities[b]		
Outer membrane: mitochondria	7.1	7.4
Outer membrane: inner membrane	15.3	35
Outer membrane: soluble	10.0	8.0

[a] The mitochondria and the mitochondrial subfractions were assayed with various amounts of each fraction (10–50 μg of protein). [b] Calculations were made by dividing the outer membrane specific activity of the acylase or monoamine oxidase by specific activity of the fraction designated.

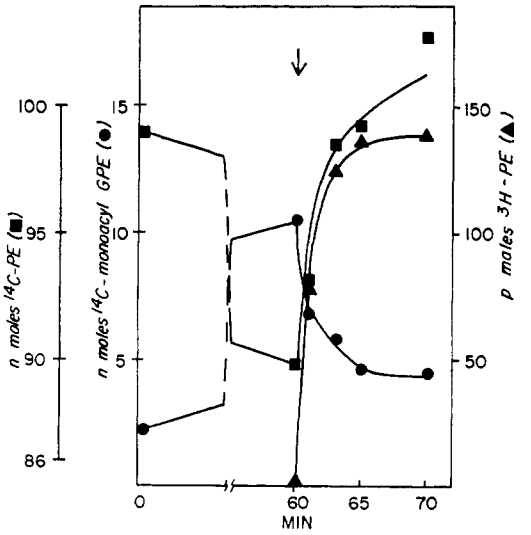

FIGURE 6: Reacylation of endogenous [^{14}C]monoacylglycerophosphorylethanolamine with [^{3}H]oleic acid. Outer membranes (2.0 mg) containing [^{14}C]ethanolamine-labeled phosphatidylethanolamine (4000 cpm) were incubated for 1 hr. EDTA (3 mmoles), the ATP solution, and [^{3}H]oleic acid (10 nmoles) were then added (the arrow) and the incubation was continued for the indicated times.

TABLE III: Acylation of 1-[^{3}H]Palmitoylglycerophosphorylethanolamine.[a]

Additions	By Outer Membranes	By Microsomes
None	0.4	1.3
Linoleoyl-CoA	8.4	12.8
Linoleic acid + ATP solution	5.2	11.5

[a] Outer membranes or microsomes (25 μg) were incubated with 30 μM 1-[^{3}H]palmitoylglycerophosphorylethanolamine and either 15 μM linoleic acid and the ATP solution or 20 μM linoleoyl-CoA. Incubations were made for 1, 3, and 5 min to show linearity of the reaction. The values reported are the average of three experiments. In nanomoles per minute per milligram of protein.

In Table II acylase and monoamine oxidase (an outer membrane marker enzyme) are compared in the mitochondrial subfractions. The highest acylase specific activity is in the outer membrane, as was the case for the monoamine oxidase and the phospholipase A (Nachbaur and Vignais, 1968; Waite, 1969). The ratio of the two enzymes in the whole mitochondria and outer membranes is the same, which shows that the acylase is located primarily in the outer membrane. However, the outer membrane to inner membrane ratio of the acylase activity is higher than that of the monoamine oxidase; possibly some acylase activity is due to inner membrane activity.

Acylation of 1-[^{3}H]palmitoylglycerophosphorylethanolamine by linoleoyl-CoA was compared with acylation by linoleic acid, ATP, and CoA to investigate whether the acyl-CoA synthetase or the acylase was rate limiting in acylation. The data in Table III show that linoleoyl-CoA was incorporated more rapidly than linoleic acid, which suggests that the acyl-CoA synthetase is probably the rate-limiting enzyme in both systems. The relative difference between the utilization of linoleoyl-CoA and the acyl-CoA synthesizing system (ATP and CoA) was apparent with the outer membranes (8.4 *vs.* 5.2). Little difference was found in the utilization of the two substrates by microsomes (12.8 *vs.* 11.5). The low level of monoacylglycerophosphorylethanolamine acylation which occurred without the addition of an acylation substrate suggests the incorporation of endogenous substrates into phosphoglycerides. The specific enzyme activities found in these experiments were higher than those found when we use [^{14}C]fatty acids, possibly because of dilution of [^{14}C]fatty acids by endogenous fatty acids. The ratio of activities found using linoleoyl-CoA and 1-[^{3}H]palmitoylglycerophosphorylethanolamine (12.8/8.4 = 1.5) is the same as that reported for [^{14}C]linoleic acid in Table I (1.7). It would appear therefore, that determination of the relative acylase activity in the two membrane fractions is not complicated by any dilution of the added substrate by endogenous compounds.

Since we found that mitochondrial outer membranes have acylase activity, we wondered why Eibl *et al.* (1969) were unable to detect acylase activity in their mitochondrial preparations. One fundamental difference between these preparations is the amount of contamination by other cell fractions (lysosomes, peroxisomes, and endoplasmic reticulum). Their preparation contained one-fifth of the total cellular acid phosphatase (a lysosomal marker enzyme) whereas the preparations used by us contained one-tenth the level of their preparations (Waite *et al.*, 1969a). The results of an experiment to test the effect of lysosomes and peroxisomes (Table IV) show that a fraction rich in lysosomes and peroxisomes ("lysosome-rich fraction") inhibit acylation in a fraction that had acylase activity when assayed alone. Increasing amounts of the lysosome-rich fraction decreased the activity to about one-quarter of that found when either the outer membranes, microsomes, or lysosome-rich fraction were assayed separately. This inhibition could be overcome partially by increasing the amount of outer membranes or microsomes. Similar results were obtained with intact mitochondria and the lysosome-rich fraction.

Acylation of Endogenous Phosphoglyceride. During a 1-hr incubation with Ca^{2+}, phospholipase A activity caused an increase of [^{14}C]ethanolamine-labeled monoacylglycerophosphorylethanolamine from 2.2 to 10.4 nmoles while [^{14}C]phosphatidylethanolamine content decreased from 99 to 90 nmoles. At 1 hr (indicated by the arrow in Figure 6) [^{3}H]oleic acid, ATP, CoA, Mg^{2+}, and albumin were added and the samples were incubated a further 1, 3, 5, and 10 min. The formation of [^{3}H,^{14}C]phosphatidylethanolamine and the decrease of [^{14}C]monoacylglycerophosphorylethanolamine proceeded at similar rates and was nearly complete after 5 min. The amount of [^{3}H]oleic acid incorporated amounted to 2.3% of the [^{14}C]monoacylglycerophosphorylethanolamine converted into [^{14}C]phosphatidylethanolamine which indicated that the acylase chiefly utilized endogenous fatty acids. The acylation reaction decreased the amount of monoacylglycerophosphorylethanolamine to the original level, but never led to complete utilization of the monoacylglycerophos-

TABLE IV: Effect of a Lysosome-Rich Fraction on Microsomal and Outer Membrane Acylation.[a]

Expt	Fraction (μg)	pmoles of Linoleic Acid Incorpd into Phosphatidyl-ethanolamine	Sum of pmoles of Linoleic Acid Incorpd into Phosphatidyl-ethanolamine	% of Sum
1-A	Outer membrane (2.5)	18		
	Outer membrane (5.0)	34		
1-B	Microsomes (5.0)	39		
	Microsomes (12.5)	140		
1-C	Lysosome (10)	49		
	Lysosome (25)	141		
2-A	Outer membrane (2.5) + lysosome (10)	57	67	85
2-B	Outer membranes (2.5) + lysosome (25)	40	159	25
2-C	Outer membrane (5.0) + lysosome (25)	82	175	47
3-A	Microsomes (5.0) + lysosome (10.0)	79	88	90
3-B	Microsomes (5.0) + lysosome (25.0)	50	180	28
3-C	Microsomes (12.5) + lysosome (25.0)	189	281	67

[a] The indicated amounts of protein were incubated 3 min with [^{14}C]linoleic acid and 1-acylglycerophosphorylethanolamine and ATP solution. The sum of picomoles of linoleic acid incorporated was calculated by adding the amount of linoleic acid incorporated by each fraction when incubated alone. The per cent of sum was calculated by dividing the amount found by the sum times 100.

phorylethanolamine. If the concentration of ATP, CoA, Mg^{2+}, or EDTA was changed, it did not alter the extent of acylation.

Earlier work showed that hydrolysis of phosphatidylethanolamine was primarily at the 2 position when mitochondria were used and at the 1 position when microsomes were used (Scherphof *et al.*, 1967). This could lead to differences in the position of fatty acid incorporation as a result of different endogenous monoacylglycerides acting as acyl acceptors. Three experimental conditions were used to study this: (1) fatty acid incorporation into endogenous phosphoglycerides without prior incubation of the membrane fractions; (2) fatty acid incorporation into phosphoglycerides after 30-min incubation of the membrane fractions with Ca^{2+}, which led to 8–10% formation of monoacyl phosphoglycerides; and (3) fatty acid incorporation into phosphoglycerides of controls incubated 30 min with EDTA (to inhibit phospholipase A), which had the same amount of monoacyl phosphoglycerides as the nonincubated fractions. Labeled fatty acids were incorporated during 10-min incubation of the membrane fractions with ATP, CoA, Mg, and albumin. The lipids were extracted and chromatographed, and the isolated phosphatidylethanolamine hydrolyzed by phospholipase A in *C. adamanteus* venom to determine the position of the labeled fatty acid. As another control, incubations were done under the same three conditions except that exogenous monoacylglycerophosphorylethanolamine was added to show that the acylation activity was not decreased as the result of the 30-min incubation. The data in Table V show that incubation of both fractions with Ca^{2+}, leading to the formation of endogenous monoacylglycerophosphorylethanolamine, caused an increase in the linoleic acid incorporation (13–38 and 7–35 pmoles incorporated by the other membrane and microsomes, respectively). The percentage of [^{14}C]linoleic acid in the 2 position dropped from 64 to 36% owing to the incubation of the microsomes. Addition of 1-acylglycerophosphorylethanolamine, however, caused 74% of [^{14}C]linoleic acid to be in the 2 position. No change in the position of incorporation effected by the outer membrane was observed upon incubation with Ca^{2+}. Both fractions incubated with EDTA prior to the acylation gave results similar to samples which had not had hydrolysis incubation. In all cases addition of monoacylglycerophosphorylethanolamine gave a severalfold increase in both the amount and per cent of incorporation into the 2 position. Although not presented here, similar experiments with [^{14}C]arachidonic acid gave the same results except that the magnitude of [^{14}C]arachidonic acid incorporation into the 1 position by microsomes was lower than that found with [^{14}C]linoleic. By contrast very little of either linoleic or arachidonic acid was incorporated into the 1 position of phosphatidylcholine under these conditions. These results show that marked qualitative differences exists between the microsomal and outer membrane enzymes of the monoacyldiacyl phosphoglyceride cycle.

Discussion

We have demonstrated that mitochondrial outer membranes possess the acylase capable of synthesizing diacyl phosphoglycerides from acyl-CoAs and monoacyl phosphoglycerides, which confirms the conclusions drawn by Stoffel and Schiefer (1968) and by Nachbaur *et al.* (1969) from studies *in vitro*. The outer membrane system differs in a number of respects from the system found in the microsomes. The saturating concentrations of the substrates was dependent upon the kind

TABLE V: Incorporation of [^{14}C]Linoleic Acid into Endogenous and Added Phosphoglyceride by Outer Membranes and Microsomes.[a]

System	Hydrolysis Incubn (30 min)	Acylation Incubn (10 min)	Product	
			Phosphatidylethanolamine (pmoles)	% [^{14}C]-Linoleic Acid in the 2 Position
Outer membranes	Not incubated	ATP	13	86
	Not incubated	ATP + monoacyl-GPE	133	94
	Incubated + Ca^{2+}	ATP	38	91
	Incubated + Ca^{2+}	ATP + monoacyl-GPE	142	93
	Incubated + EDTA	ATP	11	80
	Incubated + EDTA	ATP + monoacyl-GPE	170	92
Microsomes				
	Not incubated	ATP	7	64
	Not incubated	ATP + monoacyl-GPE	141	80
	Incubated + Ca^{2+}	ATP	35	36
	Incubated + Ca^{2+}	ATP + monoacyl-GPE	173	74
	Incubated + EDTA	ATP	9	60
	Incubated + EDTA	ATP + monoacyl-GPE	152	81

[a] Outer membranes and microsomes (2.0 g) were incubated with 2.0 mM $CaCl_2$ or 3.0 mM EDTA for the indicated lengths of time. The incubation tubes were then cooled and the ATP solution, [^{14}C]linoleic acid (50 nmoles), and, where indicated, 50 nmoles of monoacylglycerophosphorylethanolamine (GPE) were added. Also, EDTA and/or $CaCl_2$ was added so that all incubation mixtures were 2.0 mM with respect to $CaCl_2$ and 3.0 mM with respect to EDTA. Following the acylation incubation the lipids were extracted and the [^{14}C]phosphatidylethanolamine was isolated by thin-layer chromatography using chloroform–petroleum ether–acetic acid system (70:30:2) followed by the chloroform–methanol–H_2O system (65:35:4, v/v). The [^{14}C]-phosphatidylethanolamine eluted from the silicic acid was hydrolyzed by *C. Adamanteus* venom phospholipase A (specific for the 2 position), and the hydrolysis products were separated by thin-layer chromatography in the same systems. The per cent [^{14}C]-linoleic acid in the two position was calculated by dividing the amount [^{14}C]linoleic acid found after hydrolysis divided by the sum of the amount of [^{14}C]linoleic acid and the amount of [^{14}C]monoacylglycerophosphorylethanolamine times 100. The [^{14}C]-monoacylglycerophosphorylethanolamine recovered is the 1-acyl compound.

of fatty acid and monoacyl phosphoglyceride used and were different for the two-enzyme systems. These results indicate the importance of establishing optimal assay conditions with each substrate used. One of the most significant differences between the outer membrane system and the microsomal system is the position of fatty acid incorporation in phosphatidylethanolamine. Outer membranes incorporated less palmitic acid into the 2 position of phosphatidylethanolamine and less [^{14}C]linoleic acid into the 1 position of endogenous monoacylphosphatidylethanolamine than did the microsomal system. Under these conditions the acylase(s) of the microsome did not maintain the normal species specificity (1 position, saturated; 2 position, unsaturated) to the same extent as did the outer membrane acylase(s). Control experiments indicate that both outer membrane and microsomal enzymes catalyze a low level of acylation which is independent of added substrate. It is estimated that these controls were less than 10% of the reported activities and therefore indicate that the differences found between outer membranes and microsomes cannot be accounted for by a specific utilization of endogenous substrate by one or the other fraction.

Outer membranes of the mitochondria have the majority of the mitochondrial acylase activity. On the basis of comparing the distribution of marker enzymes to the distribution of the acylase it is not possible to rule out the existence of an acylase(s) in the inner membrane, where some may be present, since a low but significant phospholipase A activity was detected in the inner membrane (Waite, 1969). However, Stoffel and Schiefer (1968) concluded that the acylase is located only in the outer membrane. Also, Bygrave (1969) found in his studies *in vivo* that phospholipids of the inner membrane do not take up newly synthesized fatty acids directly. Clearly, more work is required to resolve this point. Both mitochondria and microsomes apparently have more than one acylase system. This is based on the observations that different levels of the two monoacyl derivatives are required to give maximal activity and that very little linoleic acid is incorporated into the 1 position of endogenous phosphatidylcholine (as compared with phosphatidylethanolamine) by microsomes. Also, relatively little palmitic acid is utilized for the acylation of added 1-acylglycerophosphorylcholine, compared with monoacylglycerophosphorylethanolamine.

At present, the reason for the observed acylase inhibition when the lysosome-rich fraction is incubated in combination with the mitochondria or microsomes is not clear. This

inhibition, however, might account for the failure of Eibl, Hill, and Lands to detect acylase activity in mitochondria using a color reaction based on the release of CoA from acid CoAs. We measured the incorporation of [^{14}C]fatty acids into phosphatidylethanolamine using mitochondria prepared according to their method. It was found that these mitochondrial did incorporate [^{14}C]fatty acid faster than could be accounted for by microsomal contamination. This indicates that our differences must arise from the assay procedure as well as the method of cell fractionation. If enough of a peroxisomal oxidizing enzyme were contaminating the preparation there could be an oxidation of the sulfhydryl group of CoA before it reacted with the color reagent, 5,5-dithiobis(2-nitrobenzoate). Relatively high amounts of such an enzyme (as with the added lysosome-rich fraction) would be required to observe an inhibition in our assay system since we added large amounts of CoA (250 nmoles). Such possibilities do not account for the observation that the lysosome-rich fraction has considerable acylase activity alone, but do point out the necessity of using a fraction that is as nearly pure as possible.

The microsomal specific activity found in these studies using linoleoyl-CoA and 1-[^{3}H]palmitoylglycerophosphorylethanolamine is about one-third that found by Eibl *et al.* (1969). This might be the reflection of the different assays used or of a difference in the preparation of the microsome, since the microsomes used for our studies were subjected to osmotic swelling and shrinking and sonication. This treatment was included so that direct comparison could be made between the outer membranes and the microsomes. These microsomes had extremely low acid phosphatase specific activity (about one-third that of the mitochondria and 2% that of the lysosome-rich fraction) which indicates that little if any inhibition of acylation occurred due to lysosomal contamination.

Since the mitochondria have a monoacyl phosphoglyceride–diacyl phosphoglyceride cycle, the question arises as to the function of such a cycle. One possibility would be that the monoacyl–diacyl cycle could allow for alterations in the membrane due to the differences in the physical–chemical properties of the two types of phosphoglycerides. Such alterations would cause differences in the permeability of solutes and possibly in active transport of metabolites. Changes in membrane structure were shown to accompany phospholipase A hydrolysis of mitochondrial phosphoglyceride (Waite *et al.*, 1969b). It has been postulated that mitochondrial contraction would occur upon uptake of the release fatty acid into phosphoglyceride (Wojtczak and Lehninger, 1961). Also, this cycle would give the replacement of one fatty acid on the phosphoglyceride molecule for another. In this part this might account for possible differences in the fatty acid composition of the mitochondria and microsomal phosphoglycerides. On the other hand, this cycle could not account for net accumulation of phosphoglyceride since it has been shown by Wilgram and Kennedy (1963) and McMurray and Dawson (1969) that mitochondria are incapable of synthesizing phosphatidylcholine or phosphatidylethanolamine *de novo*. It would seem, therefore, that net accumulation of phosphoglycerides must proceed *via* a transfer from the eldoplasmic reticulum.

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