

# Study of the transfer of phospholipids from the endoplasmic reticulum to the outer and inner mitochondrial membranes

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**ABSTRACT** Isolated mitochondria cannot synthesize their own phospholipids, there is only an exchange between exogenous and mitochondrial phospholipid fatty acids. In vitro, the endoplasmic reticulum phospholipids exchange with the phospholipids of the mitochondrial outer and inner membranes. Exchange of the endoplasmic reticulum phospholipids with those of the inner membrane is the same when the incubation is carried out with whole mitochondria or with mitochondria devoid of outer membranes.

**SUPPLEMENTARY KEY WORDS** phosphatidylcholine · phosphatidylethanolamine · phosphatidylinositol · microsomes · acyltransferases

IT IS NOW well established that after injection of radioactive precursors into an animal (1–3), or after incubation of radioactive precursors with a liver homogenate (4), mitochondrial phospholipids are labeled at different rates according to the type of phospholipid. It is of importance to learn whether isolated mitochondria can synthesize their own phospholipids or whether these phospholipids are synthesized elsewhere in the cell and are subsequently transferred to the mitochondria.

There are contradictory results concerning the capabilities of mitochondria to synthesize phospholipids. To study this question, several different approaches have been used, e.g., the incorporation of radioactive precursors by isolated mitochondria and the localization of phospholipid-synthesizing enzymes.

Using radioactive precursors containing  $^{32}\text{P}$ , Wilgram and Kennedy (5) found no incorporation into phospholipids of the mitochondria. On the other hand, Gar-

bus, DeLuca, Loomans, and Strong (6), Galliard and Hawthorne (7), Hajra and Agranoff (8), and Zborowski and Wojtczak (9) have shown that the main phospholipids, i.e., phosphatidylcholine and phosphatidylethanolamine, were not labeled when mitochondria were incubated with  $^{32}\text{P}$ , although there was some incorporation of radioactivity into phosphatidic acid, diphosphoinositide, and acyl dihydroxyacetone phosphate. In studies in which choline- $^{14}\text{C}$  or ethanolamine- $^{14}\text{C}$  were used as precursors, there were also contradictory results. McMurray and Dawson (10) observed no incorporation of these precursors into phospholipids, although Bygrave and Kaiser (11) reported that there was incorporation. McMurray and Dawson (10) showed that there was no incorporation of inositol- $^{14}\text{C}$  into phospholipids, while Kaiser (12) demonstrated that serine- $^{14}\text{C}$  and glycerophosphate- $^{14}\text{C}$  were incorporated. In a recent paper Jungalwala and Dawson (13) stated that mitochondria entirely devoid of microsomal contamination cannot synthesize phosphatidylcholine.

Investigators who have studied the localization of phospholipid-synthesizing enzymes have reported different results. Although it is well established (5) that microsomes have all the enzymes for phospholipid synthesis, only Stoffel and Schiefer (14) found them in mitochondria. The presence of  $\alpha$ -glycerolphosphate acyltransferase (2.3.1.15) (15–17) and diglyceride acyltransferase (2.3.1.20) (5, 15, 18–20) in mitochondria has been reported, although Lands (21) and Eibl, Hill, and Lands (22) believe that these enzymes are not in mitochondria.

In the present work we have studied the incorporation of sodium phosphate- $^{32}\text{P}$ , glycerol-1- $^{14}\text{C}$ , and palmitate-1- $^{14}\text{C}$  into phospholipids by isolated mitochondria. The

results show that phospholipids are labeled only when the mitochondria are incubated with the labeled palmitate. We have concluded that isolated mitochondria cannot synthesize phospholipids, but are able only to exchange exogenous fatty acids with mitochondrial phospholipid fatty acids. This is in agreement with studies on the localization of diglyceride acyltransferase in mitochondria (5, 15, 18–20).

We therefore believe that mitochondrial phospholipids are synthesized elsewhere in the cell and are then transferred to the mitochondria. Wirtz and Zilversmit (23) and McMurray and Dawson (10) suggested that they could originate from microsomal phospholipids and they studied the transfer of phospholipids from the microsomal fraction to whole mitochondria possessing both outer and inner membranes. In the present work we studied the exchange of endoplasmic reticulum phospholipids with those of the mitochondrial outer and inner membranes. The two membranes were isolated after incubation of the whole mitochondria with the endoplasmic reticulum. In order to define the role of the outer membrane in the phospholipid transfer mechanism, we also carried out studies in which labeled microsomes were incubated with mitochondria devoid of their outer membrane.

## MATERIALS AND METHODS

### *Preparation of Subcellular Fractions of Rat Liver*

Mitochondria were prepared by the technique of Harel, Jacob, and Moulé (24). The single-membrane mitochondria were obtained by the technique of Lévy, Toury, and André (25). The microsomal fraction was prepared by the technique of Dallner, Siekevitz, and Palade (26). The mitochondrial membranes were separated by the technique of Lévy et al. (25). The supernatant fraction which was utilized in transfer experiments was obtained after total elimination of the microsomes by the technique of Wirtz and Zilversmit (23).

### *Preparation of Labeled Microsomes*

Rats were injected intraperitoneally with sodium phosphate- $^{32}\text{P}$  (330  $\mu\text{Ci}/200\text{-g}$  rat) or glycerol- $1\text{-}^{14}\text{C}$  (200  $\mu\text{Ci}/200\text{-g}$  rat). The animals were killed 15 hr after injection of the sodium phosphate- $^{32}\text{P}$  and 30 min after the glycerol injection. The microsomes were then isolated (26).

### *Incubation Medium*

For the study of phospholipid synthesis by isolated mitochondria the basic incubation medium was as follows: Tris buffer pH 7.4, 80 mM; phosphate buffer pH 7.4, 20 mM; extracted mitochondrial fatty acids, 3 mM; serine, 1.5 mM; sodium succinate, 10 mM;  $\text{MgCl}_2$ ,

2 mM; glycerol, 1.5 mM; ATP, 19 mM; methionine, 1.5 mM. 15 ml of this medium was added to 5 ml of mitochondrial suspension which contained 500 mg of mitochondrial proteins in 0.25 M sucrose. Such a preparation of mitochondria is capable of carrying out oxidative phosphorylation.

500  $\mu\text{Ci}$  of sodium phosphate- $^{32}\text{P}$ , 16  $\mu\text{Ci}$  of sodium palmitate- $1\text{-}^{14}\text{C}$ , or 50  $\mu\text{Ci}$  of glycerol- $1\text{-}^{14}\text{C}$  was added, and the mitochondria were incubated for 20 min at  $30^\circ\text{C}$ .

In the study of transfer of phospholipid from microsomes to mitochondrial membranes, the incubation medium was as follows: 1 ml of a suspension of mitochondria (corresponding to 120 mg of proteins), 1 ml of a suspension of microsomes (corresponding to 80 mg of proteins), and 60 ml of microsomal supernatant.

In the studies of the transfer of microsomal phospholipids to mitochondria with a single membrane, preparations of whole mitochondria were replaced by an equivalent amount of mitochondria with a single membrane.

In the last two studies the incubations were for 20 min at  $30^\circ\text{C}$ . After incubation the mitochondria were isolated by differential centrifugation at 8500  $g$  for 15 min. They were washed with 0.25 M sucrose to remove any contaminating microsomes. The microsomal contamination was about 1% as judged by assay of NADPH-cytochrome c reductase, a specific microsomal marker enzyme.

### *Phospholipid Isolation and Measure of Specific Radioactivity*

The phospholipids were isolated and their concentration was measured by techniques previously described by Lévy and Sauner (27). Due to the minute quantities of biological material available, it was impossible to separate certain minor phospholipids from the major phospholipids. We were therefore obliged to isolate phosphatidylethanolamine and phosphatidylserine together, and phosphatidic acid and cardiolipin together. In some studies phosphatidylcholine was counted with phosphatidylinositol.

Radioactivity was measured by liquid scintillation counting (Packard Liquid Scintillation Spectrometer, model 3214).

## RESULTS AND DISCUSSION

### *Phospholipid Biosynthesis by Isolated Mitochondria*

The results of this study are summarized in Table 1. They show that fatty acids, but not glycerol or inorganic phosphate, are incorporated into phospholipids. The maximum incorporation of palmitate- $1\text{-}^{14}\text{C}$  was in sphingomyelin, which is not surprising in view of the

TABLE 1 IN VITRO INCORPORATION OF RADIOACTIVE PRECURSORS INTO THE MITOCHONDRIAL PHOSPHOLIPIDS

	Ortho-phosphate- <sup>32</sup> P	Glycerol-1- <sup>14</sup> C	Palmitate-1- <sup>14</sup> C
	cpm/μg of phosphorus		
Lysophosphatidylcholine	6.6	4.5	4,312
Sphingomyelin	11.0	14.6	101,600
Phosphatidylcholine + phosphatidylinositol	0.73	1.2	4,460
Phosphatidylethanolamine + phosphatidylserine	0.79	0.6	3,835
Phosphatidic acid + cardiolipin	0.91	2.6	1,060

TABLE 2 EFFECT OF COFACTORS ON THE INCORPORATION OF ORTHOPHOSPHATE-<sup>32</sup>P INTO PHOSPHOLIPIDS BY ISOLATED MITOCHONDRIA

	Additions to the Basic Medium				
	None	CTP*	CDP-Choline*	Phosphoryl-serine†	Phosphoryl-ethanolamine†
	cpm/μg of phosphorus				
Lysophosphatidylcholine	6.6	2.7	4.9	0.9	1.4
Sphingomyelin	11.0	1.1	9.2	6.6	0
Phosphatidylcholine + phosphatidylinositol	0.7	1.0	0.3	0.2	0.4
Phosphatidylethanolamine + phosphatidylserine	0.8	0.4	0.3	0.3	0.3
Phosphatidic acid + cardiolipin	0.9	1.2	0.6	0.4	1.1

\* Final concentration, 0.5 mM.

† Final concentration, 1.5 mM.

TABLE 3 TRANSFER OF PHOSPHOLIPIDS FROM THE <sup>32</sup>P-LABELED MICROSOMES TO THE OUTER AND INNER MITOCHONDRIAL MEMBRANES

	Microsomes before Incubation	Outer Mitochondrial Membrane	Inner Mitochondrial Membrane
	cpm/μg of phosphorus		
Lysophosphatidylcholine	113	45	210
Sphingomyelin	86	155	60
Phosphatidylcholine	560	320	280
Phosphatidylinositol	480	158	43
Phosphatidylethanolamine + phosphatidylserine	502	192	74
Phosphatidic acid + cardiolipin	210	54	7

relatively high content of saturated fatty acids in this phospholipid.

The effects of CTP, CDP-choline, phosphorylethanolamine and phosphorylserine on the incorporation of inorganic <sup>32</sup>P were also studied. The data in Table 2 show that these compounds, which are intermediates in

phospholipid synthesis, did not affect the incorporation of <sup>32</sup>P into phospholipids.

We verified that the fatty acid radioactivity represents a true incorporation, and not an adsorption of the palmitic acid on phospholipid molecules, by extracting phospholipids from the same medium without incubation at zero time; there was no radioactivity in the phospholipids.

We can therefore conclude that, under our experimental conditions, isolated mitochondria cannot synthesize phospholipids de novo. However, the fatty acids of the phospholipids do exchange with "exogenous" fatty acids. This observation is in agreement with the fact that isolated mitochondria possess their own acyltransferases.

### Phospholipid Transfer Study between Microsomes and Mitochondrial Membranes

If microsomes and mitochondria are incubated together as previously described, there is a transfer of phospholipids between the microsomes and the outer and inner mitochondrial membranes. Table 3 shows results of studies in which microsomal phospholipids labeled with sodium phosphate-<sup>32</sup>P were incubated with mitochondria. Table 4 shows results of similar studies in which microsomes labeled with glycerol-1-<sup>14</sup>C were used. They represent the mean of three experiments.

Taking into consideration both the total amount of phospholipids present and their specific activities, we can estimate the degree of transfer for each of the membranes studied. The calculation was not done for lysophosphatidylcholine and sphingomyelin, since the percentage error<sup>1</sup> was too high (50–100%) due to the very

<sup>1</sup> The level of error is expressed in %, calculated from the value of

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

TABLE 4 TRANSFER OF PHOSPHOLIPIDS FROM THE GLYCEROL-1-<sup>14</sup>C-LABELED MICROSOMES TO THE OUTER AND INNER MITOCHONDRIAL MEMBRANES

	Microsomes before Incubation	Outer Mitochondrial Membrane	Inner Mitochondrial Membrane
	<i>cpm/μg of phosphorus</i>		
Lysophosphatidylcholine	29	24	9
Sphingomyelin	17	65	11
Phosphatidylcholine	147	72	62
Phosphatidylinositol	84	21	6
Phosphatidylethanolamine + phosphatidylserine	378	85	28
Phosphatidic acid + cardiolipin	214	132	19

TABLE 5 TRANSFER OF PHOSPHOLIPIDS FROM THE <sup>32</sup>P-LABELED MICROSOMES TO THE SINGLE-MEMBRANE MITOCHONDRIA

	Microsomes before Incubation	Inner Mitochondrial Membrane
	<i>cpm/μg of phosphorus</i>	
Lysophosphatidylcholine	229	100
Sphingomyelin	185	64
Phosphatidylcholine	885	544
Phosphatidylinositol	567	129
Phosphatidylethanolamine + phosphatidylserine	1001	143
Phosphatidic acid + cardiolipin	—	26

TABLE 6 TRANSFER OF PHOSPHOLIPIDS FROM THE GLYCEROL-1-<sup>14</sup>C-LABELED MICROSOMES TO THE SINGLE-MEMBRANE MITOCHONDRIA

	Microsomes before Incubation	Inner Mitochondrial Membrane
	<i>cpm/μg of phosphorus</i>	
Lysophosphatidylcholine	29	23
Sphingomyelin	17	28
Phosphatidylcholine	147	81
Phosphatidylinositol	84	4
Phosphatidylethanolamine + phosphatidylserine	378	15
Phosphatidic acid + cardiolipin	214	8

small quantities of these phospholipids in the membranes. For phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, the calculated error was only 10% in the different experiments, and we were able to make this calculation.

The degree of transfer was phosphatidylethanolamine ≥ phosphatidylcholine > phosphatidylinositol. For the outer membrane phospholipids, the transfer was about 13–20% for phosphatidylethanolamine, 15% for phosphatidylcholine, and 8% for phosphatidylinositol. For the inner membrane, it was 34% for phosphatidyl-

ethanolamine, 25% for phosphatidylcholine, and 4–8% for phosphatidylinositol. These calculations were based on data from studies in which microsomal phospholipids were labeled with inorganic phosphate-<sup>32</sup>P. Similar results were obtained in studies with glycerol-1-<sup>14</sup>C. The results indicate that the whole phospholipid molecule is transferred from microsomes to mitochondrial membranes. Tables 5 and 6 summarize the results obtained in experiments in which the microsomes were incubated with single-membrane mitochondria. The calculations show that the phospholipid transfer was of the same magnitude.

We can conclude, in agreement with Wirtz and Zilversmit (23) and McMurray and Dawson (10), that, while isolated mitochondria cannot synthesize their own phospholipids, these phospholipids could originate from microsomes.

In addition, our present work shows that there is the same degree of transfer to each of the two mitochondrial membranes, and that the transfer of phospholipids to the inner membrane does not depend on the presence of the outer membrane.

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